

**STRUCTURAL AND FUNCTIONAL PROPERTIES OF LUPIN PROTEINS AND
BIOACTIVE PEPTIDES**

By

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ABSTRACT

This thesis investigated the structural, physicochemical, and techno-functional properties of white lupin (*Lupinus albus*) and blue lupin (*Lupinus angustifolius*) proteins across multiple fractionation levels and explored enzymatic strategies to enhance functionality and generate bioactive peptides. Protein isolates were produced by alkaline extraction and isoelectric precipitation, followed by solubility-based fractionation into albumin, globulin, and glutelin classes, with globulins further separated into vicilin (7S) and legumin (11S) sub-fractions. All fractions were characterized for molecular structure, amino acid composition, and techno-functional properties at pH 3–9. Both isolates had similar essential amino acid profiles, with sulfur-containing amino acids, valine, and lysine being limiting, and achieved emulsifying performance comparable to soybean protein isolate at neutral and alkaline pH. Higher α -helix-to- β -sheet ratios at pH 7 and 9 were associated with smaller oil droplet sizes and improved emulsion stability. Fractionation revealed that isolate-level behavior masks the contributions of individual components: white lupin globulin and vicilin were uniquely not limited in sulfur-containing amino acids (107% and 127% amino acid scores), blue lupin vicilin emerged as the most versatile fraction with high emulsion stability (95–100%) and foaming capacity (75–85%), and white lupin legumin exhibited the highest thermal stability and lowest gelation concentration (8%). Transglutaminase-catalyzed cross-linking (10–15 U/g) enhanced emulsifying properties at pH 3 by inducing structural rearrangements, whereas excessive cross-linking (20–25 U/g) compromised functionality. The bioactive potential was further explored by generating arginase-inhibitory peptides. White lupin flavourzyme hydrolysate showed the highest inhibitory activity (~35% at 800 μ g/mL). Peptide purification yielded ten sequences, with DMGAVA and VVALKKVGVLLKKPGPT showing the highest inhibition. Kinetic analysis demonstrated mixed-type inhibition, with DMGAVA affecting both K_m and V_{max} (K_i ~266 μ M).

and VVALKKVGVLKKPGPT exhibiting predominantly competitive behavior ($K_i \sim 589 \mu\text{M}$). A complementary *in-silico* strategy generated 24 candidate peptides; of these, DQR exhibited the highest inhibitory activity (17% at $20 \mu\text{g/mL}$) with mixed-type inhibition. Discrepancies between computational predictions and experimental outcomes highlighted the limitations of binding affinity as a sole predictor of inhibitory function. Collectively, this thesis establishes structure-function relationships governing lupin protein functionality and identifies lupin proteins as a promising, underexplored source of bioactive peptides for functional food development.

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FOREWARD

This doctoral thesis was prepared in a manuscript format consisting of eight manuscripts, following the General Introduction. The manuscripts were prepared according to the style guidelines of their respective journals: Manuscript 1 (*Food Reviews International*), Manuscript 2 (*ACS Food Science and Technology*), Manuscript 3 (*LWT – Food Science and Technology*), Manuscript 4 (*Sustainable Food Proteins*), Manuscript 5 (*Food Chemistry: X*), Manuscript 6 (*Applied Food Research*), Manuscript 7 (*Journal of Functional Foods*), and Manuscript 8 (*Food Chemistry: Molecular Sciences*). Manuscripts 1, 2, 3, 4, 5, 6, and 8 have been published, while Manuscript 7 has been submitted and is under review in the *Journal of Functional Foods*. A transition statement at the end of each manuscript chapter links it to the subsequent chapter, providing coherence. Finally, the last chapter (Chapter 10) contains an overall summary of the work, limitations, and possible future directions.

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Figure 7.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of transglutaminase polymerized white (A) and blue (B) lupin protein isolates. **1.** Protein standard marker (kDa); **2.** WT25U; **3.** WT20U; **4.** WT15U; **5.** WT10U; **6.** WT5U; **7.** WLPI; **8.** Protein standard marker (kDa). **9.** Protein standard marker (kDa); **10.** BT25U; **11.** BT20U; **12.** BT15U; **13.** BT10U; **14.** BT5U; **15.** BLPI; **16.** Protein standard marker (kDa). WLPI-white lupin protein isolate; WT5U-white transglutaminase 5 units; WT10U-white transglutaminase 10 units; WT15U-white transglutaminase 15 units; WT20U-white transglutaminase 20 units; WT25U-white

transglutaminase 25 units; BLPI-blue lupin protein isolate; BT5U-Blue transglutaminase 5 units; BT10U-Blue transglutaminase 10 units; BT15U-blue transglutaminase 15 units; BT20U-blue transglutaminase 20 units; BT25U-blue transglutaminase 25 units

Figure 7.3 Secondary structure analysis from FTIR spectra of transglutaminase polymerized white (A) and blue (B) lupin protein isolates. WLPI-white lupin protein isolate; WT5U-white transglutaminase 5 units; WT10U-white transglutaminase 10 units; WT15U-white transglutaminase 15 units; WT20U-white transglutaminase 20 units; WT25U-white transglutaminase 25 units; BLPI-blue lupin protein isolate; BT5U-Blue transglutaminase 5 units; BT10U-Blue transglutaminase 10 units; BT15U-blue transglutaminase 15 units; BT20U-blue transglutaminase 20 units; BT25U-blue transglutaminase 25 units

Figure 7.4 Effect of pH and enzyme concentration on emulsification capacity (A) and emulsion stability (B) of transglutaminase polymerized lupin protein isolates. Values with different superscripts within the same pH are significantly different ($p < 0.05$) as determined by two-way ANOVA and Duncan's multiple range test. WLPI-white lupin protein isolate; WT5U-white transglutaminase 5 units; WT10U-white transglutaminase 10 units; WT15U-white transglutaminase 15 units; WT20U-white transglutaminase 20 units; WT25U-white transglutaminase 25 units; BLPI-blue lupin protein isolate; BT5U-Blue transglutaminase 5 units; BT10U-Blue transglutaminase 10 units; BT15U-blue transglutaminase 15 units; BT20U-blue transglutaminase 20 units; BT25U-blue transglutaminase 25 units

Figure 7.5 Confocal laser scanning microscopy of lupin protein isolates and transglutaminase polymerized lupin protein isolates. The scale bars within the figures represent 40 μm in length. WLPI-white lupin protein isolate; WT5U-white transglutaminase 5 units; BLPI-blue lupin protein isolate; BT5U-blue transglutaminase 5 units

Figure 7.6 Effect of pH and enzyme concentration on A: flocculation index and B: coalescence index of transglutaminase polymerized lupin protein isolates. Values with different superscripts within the same pH are significantly different ($p < 0.05$) as determined by two-way ANOVA and Duncan's multiple range test. WLPI-white lupin protein isolate; WT5U-white transglutaminase 5 units; WT10U-white transglutaminase 10 units; WT15U-white transglutaminase 15 units; WT20U-white transglutaminase 20 units; WT25U-white transglutaminase 25 units; BLPI-blue lupin protein isolate; BT5U-Blue transglutaminase 5 units; BT10U-Blue transglutaminase 10 units; BT15U-blue transglutaminase 15 units; BT20U-blue transglutaminase 20 units; BT25U-blue transglutaminase 25 units

Figure 8.1 (A) Lupin protein hydrolysate arginase inhibition (B) First round of HPLC separated fractions (C) Arginase inhibitory activity of the first round of HPLC separated fractions (D) Second round of HPLC separated fractions (E) Arginase inhibitory activity of the second round of HPLC separated fractions (F) Arginase inhibitory activities of synthesized peptide fractions.

Key: WCHY-White Chymotrypsin Hydrolysate, BCHY- Blue Chymotrypsin Hydrolysate, WTRP- White Trypsin Hydrolysate, BTRP- Blue Trypsin Hydrolysate, WALC-White Alcalase Hydrolysate, BALC- Blue Alcalase Hydrolysate, WPEP-White Pepsin Hydrolysate, BPEP- Blue Pepsin Hydrolysate, WPEP-PAN- White Pepsin Pancreatin Hydrolysate, BPEP-PAN- Blue Pepsin Pancreatin Hydrolysate. SF1-1 (KGPPSGSATPT), SF1-2 (DMGAVA), SF1-3 (VVALKKVGV LKKPGPT), SF5-1 (PPSVGY), SF6-1 (SGKVTSFT), SF6-2 (THGRLSVP), SF7-1 (VLAKGF), SF7-2 (LLGCPTYG), SF7-3 (RAKKLVLRVGVPP), SF7-4 (HCLSFST).

Figure 8.2 Kinetics of arginase inhibition (Lineweaver-Burk plot) of the synthesized peptides (A). SF1-2 (DMGAVA) (B). SF1-3 (VVALKKVGVLLKKPGPT).

Figure 8.3 Molecular docking binding interactions of A. SP1-2 (DMGAVA) and B. SP1-3 (VVALKKVGVLLKKPGPT). Radius of gyration (R_g) of C. SP1-2 (DMGAVA) and D. SP1-3 (VVALKKVGVLLKKPGPT).

Figure 8.4 Root mean square deviation of A. SP1-2 (DMGAVA) and B. SP1-3 (VVALKKVGVLLKKPGPT). Root mean square fluctuation of C. SP1-2 (DMGAVA) and D. SP1-3 (VVALKKVGVLLKKPGPT).

Figure 9.1 Chemical structure of chemical compounds (DB series) and synthetic peptide (REGNH) targeting arginase I and II

Figure 9.2 Docking interaction of (1) **CQR** with arginase I (PDB ID: 2AEB). (2) **CQR** with arginase II (PDB ID: 4IXU). (3) **DQR** with arginase I (PDB ID: 2AEB). (4) **DQR** with arginase II (PDB ID: 4IXU)

Figure 9.3 Docking interaction of (5) **DR** with arginase I (PDB ID: 2AEB). (6) **DR** with arginase II (PDB ID: 4IXU). (7) **QER** with arginase I (PDB ID: 2AEB). (8) **QER** with arginase II (PDB ID: 4IXU)

Figure 9.4 Docking interaction of (9) DB03144 with arginase I (PDB ID: 2AEB). (10) DB03144 with arginase II (PDB ID: 4IXU). (11) REGNH with arginase I (PDB ID: 2AEB). (12) REGNH with arginase II (PDB ID: 4IXU)

Figure 9.5 RMSD of arginase I and arginase II on binding with (A) CQR. (B) DR (C) DQR. Color key: blue-Protein; red-Ligand

Figure 9.6 RMSD of arginase I and arginase II on binding with (D) QER. (E) DB03144. (F) REGNH. Color key: blue-Protein; red-Ligand

Figure 9.7 RMSF of arginase I and arginase II on binding with (A) CQR (B) DR (C) DQR

Figure 9.8 RMSF of arginase I and arginase II on binding with (D) QER (E) DB03144 (F) REGNH

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Figure 9.11 (A) In vitro arginase inhibitory properties of CQR, DR, DQR, QER, and REGNH peptides. Lineweaver-Burk plots showing kinetics of arginase inhibition by (B) DQR, (C) QER, and (D) REGNH peptides

LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ADME	Absorption, distribution, metabolism, and excretion
AE-IEP	Alkaline extraction–isoelectric precipitation
ANOVA	Analysis of variance
ANS	8-Anilinonaphthalene-1-sulfonic acid
ARG-1	Arginase I (cytosolic isoform)
ARG-2	Arginase II (mitochondrial isoform)
ATR-FTIR	Attenuated total reflectance Fourier-transform infrared spectroscopy
BALC	Blue lupin alcalase hydrolysate
BBB	Blood–brain barrier
BBi	Bowman–Birk inhibitor
BCAA	Branched-chain amino acids
BCHY	Blue lupin chymotrypsin hydrolysate
BEC	S-(2-Boronoethyl)-L-cysteine
BLA1b	Blue lupin albumin
BLGlo	Blue lupin globulin
BLGlu	Blue lupin glutelin
BLLEG	Blue lupin legumin
BLPI	Blue lupin protein isolate
BLVL	Blue lupin vicilin

BPEP	Blue lupin pepsin hydrolysate
BPEP-PAN	Blue lupin pepsin–pancreatin hydrolysate
BT5U–BT25U	Blue lupin transglutaminase-treated (5–25 U/g protein)
BTRP	Blue lupin trypsin hydrolysate
CD	Circular dichroism
CI	Coalescence index
CLSM	Confocal laser scanning microscopy
CQR	Cys-Gln-Arg (synthesized peptide)
d _{3,2}	Surface-weighted mean diameter (Sauter mean diameter)
D _{4,3}	Volume-weighted mean diameter
DDW	Double-distilled water
DMGAVA	Asp-Met-Gly-Ala-Val-Ala (synthesized peptide)
DPP-IV	Dipeptidyl peptidase-IV
DQR	Asp-Gln-Arg (synthesized peptide)
DR	Asp-Arg (synthesized peptide)
DSC	Differential scanning calorimetry
DTT	Dithiothreitol
EC	Emulsifying capacity
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
ES	Emulsion stability

FC	Foaming capacity
FI	Flocculation index
FPLC	Fast protein liquid chromatography
FS	Foam stability
FTIR	Fourier-transform infrared spectroscopy
GCLM	Glutamate–cysteine ligase modifier subunit
GI	Gastrointestinal
GPX	Glutathione peroxidase
GRAS	Generally recognized as safe
HCl	Hydrochloric acid
HDL	High-density lipoprotein
HPLC	High-performance liquid chromatography
IC ₅₀	Half-maximal inhibitory concentration
IEP	Isoelectric precipitation
IVPD	In vitro protein digestibility
k _{cat}	Catalytic rate constant
kDa	Kilodalton
K _i	Inhibition constant
K _m	Michaelis constant
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
LDL	Low-density lipoprotein

LGC	Least gelation concentration
MD	Molecular dynamics
MMGBSA	Molecular mechanics—generalized Born surface area
MPa	Megapascal
Mr	Relative molecular mass
MS	Mass spectrometry
mTGase	Microbial transglutaminase
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NO	Nitric oxide
nor-NOHA	N ω -Hydroxy-L-arginine
OHC	Oil holding capacity
OPA	o-Phthaldialdehyde
P-gp	P-glycoprotein
PBMC	Peripheral blood mononuclear cells
PDB	Protein Data Bank
QER	Gln-Glu-Arg (synthesized peptide)
REGNH	Arg-Glu-Gly-Asn-His (synthesized peptide)
Rg	Radius of gyration
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation

ROS	Reactive oxygen species
RP-HPLC	Reverse-phase high-performance liquid chromatography
rpm	Revolutions per minute
SASA	Solvent-accessible surface area
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEA	Similarity Ensemble Approach
SH	Sulfhydryl (free thiol) group
SMILES	Simplified Molecular Input Line Entry Specification
SOD	Superoxide dismutase
SPI	Soybean protein isolate
SS	Disulfide bond
TCA	Trichloroacetic acid
TG	Transglutaminase
TPSA	Total polar surface area
Tris	Tris(hydroxymethyl)aminomethane
UF	Ultrafiltration
UV	Ultraviolet
v/v	Volume per volume
V _{max}	Maximum reaction velocity
VVALKKVGVLKKPGPT	Val-Val-Ala-Leu-Lys-Lys-Val-Gly-Val-Leu-Lys-Lys-Pro-Gly-Pro-Thr (synthesized peptide)

w/v	Weight per volume
WALC	White lupin alcalase hydrolysate
WCHY	White lupin chymotrypsin hydrolysate
WHC	Water holding capacity
WLA1b	White lupin albumin
WLGlo	White lupin globulin
WLGlu	White lupin glutelin
WLLEG	White lupin legumin
WLPI	White lupin protein isolate
WLVL	White lupin vicilin
WPEP	White lupin pepsin hydrolysate
WPEP-PAN	White lupin pepsin–pancreatin hydrolysate
WT5U–WT25U	White lupin transglutaminase-treated (5–25 U/g protein)
WTRP	White lupin trypsin hydrolysate

CHAPTER ONE

1.1 General Introduction

The global demand for dietary protein is projected to rise sharply as the world population approaches 9.7 billion by 2050 (United Nations Department of Economic and Social Affairs, 2019). Meeting this demand through conventional animal agriculture alone is increasingly untenable because livestock production accounts for a disproportionate share of agricultural greenhouse-gas emissions, land use, and freshwater consumption (Poore and Nemecek, 2018). These environmental pressures, combined with growing consumer awareness of ethical and health considerations, have intensified the search for high-quality plant-based protein alternatives. Among the candidate crops, legumes occupy a privileged position because of their capacity for symbiotic nitrogen fixation, which simultaneously enriches soil fertility and yields seeds with protein content that rivals or exceeds that of many cereals and oilseeds. Within the legume family, lupin (*Lupinus* spp.) remains comparatively underutilized despite several agronomic and nutritional advantages. Dehulled lupin seeds contain 39–53% protein on a dry-matter basis, placing them among the most protein-dense legumes and approaching the levels found in soybean (Vogelsang-O'Dwyer *et al.*, 2020). Unlike soybeans, lupins have negligible starch content, are rich in dietary fiber, and contain appreciable levels of lipids, a combination that broadens their potential in food formulation (Shrestha *et al.*, 2021).

Of the roughly 200 species in the genus, white lupin (*Lupinus albus*) and narrow-leafed (blue) lupin (*Lupinus angustifolius*) are the two species of greatest commercial and research interest, and their proteins form the focus of the present work. Together, *L. angustifolius* and *L. albus* account for the majority of global lupin production for food and feed. *L. angustifolius* is the most widely cultivated lupin species worldwide: it is the dominant species in Australia, the world's largest lupin

producer, and is also grown extensively across Germany and Eastern Europe (Vogelsang-O'Dwyer *et al.*, 2020). *L. albus*, in turn, is the principal species traditionally cultivated in the Mediterranean basin and remains important in southern Europe, where its distinct protein composition is valued. Both species also benefit from well-established sweet, low-alkaloid cultivars developed through decades of breeding, which are essential for human consumption because the quinolizidine alkaloids present in wild lupins are toxic (Van de Noort, 2024). Although both are protein-rich, they differ in the relative abundance of their conglutins and in their physicochemical properties, making them well suited for comparative structure–function investigations. By contrast, the other cultivated lupin species were excluded primarily for practical reasons. *L. luteus* (yellow lupin), although high in protein, is cultivated on a considerably smaller commercial scale. *L. mutabilis* (Andean lupin) contains higher quinolizidine alkaloid concentrations, requires extensive debittering before consumption, and remains largely confined to the Andean region with limited international supply chains (Boukid & Pasqualone, 2022).

Lupin seed storage proteins are classified under the Osborne system as globulins, albumins, prolamins, and glutelins, with globulins dominating albumins in an approximately 9:1 ratio (Duranti *et al.*, 2008). The globulin fraction comprises four conglutins: α (legumin-type, 11S), β (vicilin-type, 7S), γ (a basic 7S protein), and δ (a sulfur-rich 2S albumin), first resolved by Blagrove and Gillespie (1975). Together, α - and β -conglutin account for roughly 80% of total seed protein, although their relative proportions differ between *L. albus* and *L. angustifolius* (Duranti *et al.*, 2008; Shrestha *et al.*, 2021). While whole protein isolates are the most common form in which lupin proteins have been studied, they are complex mixtures whose behavior reflects the weighted average of several structurally distinct components. Evidence suggests that fractionating isolates into their constituent classes, albumins, globulins, and glutelins on the basis of solubility,

or vicilins (7S) and legumins (11S) on the basis of sedimentation coefficients, can reveal functional attributes, which may not be fully apparent when proteins are evaluated at the whole-isolate level (Boye *et al.*, 2010). For example, 7S vicilins lack disulfide bridges and tend to be more flexible at interfaces, whereas 11S legumins possess interchain disulfide bonds that confer greater thermal stability but lower molecular mobility. These structural contrasts are expected to translate into measurable differences in emulsifying capacity, foam formation, gelation, and water-holding behavior. Systematic characterization of individual fractions from both *L. albus* and *L. angustifolius* is, therefore, essential for identifying which components are best suited to specific food applications.

Among the techno-functional properties relevant to food systems, emulsification occupies a central position because protein-stabilized oil-in-water emulsions are found in diverse products, including salad dressings, beverages, infant formulas, and processed meats. The ability of a protein to lower interfacial tension, form a cohesive viscoelastic film around oil droplets, and resist coalescence over storage depends on a combination of surface hydrophobicity, charge density, molecular flexibility, and secondary-structure composition (McClements, 2015). These determinants vary not only among protein sources but also with environmental conditions such as pH, ionic strength, and temperature. Early comparative data suggest that lupin protein isolates can achieve emulsifying performance broadly comparable to that of soybean protein isolates (Burgos-Díaz *et al.*, 2016), yet the responses of individual lupin fractions and enzymatically modified lupin proteins to processing conditions remain poorly characterized. One strategy for enhancing the emulsifying and gelling performance of food proteins is enzymatic cross-linking using microbial transglutaminase (EC 2.3.2.13). This enzyme catalyzes an acyl-transfer reaction between the γ -carboxamide group of peptide-bound glutamine and the ϵ -amino group of lysine, forming covalent

ϵ -(γ -glutamyl)lysine isopeptide bonds that can link subunits within a molecule or bridge adjacent polypeptide chains (Mattice and Marangoni, 2021). The formation of such polymeric networks has been shown to enhance gel strength, emulsion stability, and thermal stability in dairy, soy, and pea protein systems (Mohammad et al., 2018). However, whether lupin proteins—which differ from soy and pea proteins in subunit composition, glycosylation status, and the availability of surface-accessible lysine and glutamine residues—are equally amenable to transglutaminase-catalyzed polymerization remains unclear. Elucidating the relationship between the extent of cross-linking and the resulting structural and functional changes in both *Lupinus albus* and *Lupinus angustifolius* protein isolates is, therefore, important for improving their functional performance and expanding their applications in food systems.

Enzymatic treatment of lupin proteins can serve a second, complementary purpose beyond structural modification: controlled proteolysis using food-grade enzymes releases peptide fragments whose amino acid sequences may confer biological activities that are not present in the intact protein. Bioactive peptides derived from legume sources have attracted considerable research attention for their antihypertensive, antioxidant, anti-inflammatory, and enzyme-inhibitory properties (Lammi et al., 2019). However, limited information is available on peptides that inhibit arginase, a metalloenzyme whose dysregulation is implicated in a range of cardiovascular and inflammatory disorders. Arginase (EC 3.5.3.1) is a binuclear manganese-dependent enzyme that catalyzes the hydrolysis of L-arginine to L-ornithine and urea. Two mammalian isoforms exist: cytosolic ARG-1, expressed predominantly in hepatocytes as part of the urea cycle, and mitochondrial ARG-2, which is more widely distributed across tissues including the vasculature, kidneys, and immune cells (Borek et al., 2020). Because arginase and endothelial nitric oxide synthase compete for the same substrate, elevated arginase activity diverts

L-arginine away from nitric oxide production, contributing to endothelial dysfunction, vasoconstriction, and downstream pathologies such as hypertension, atherosclerosis, and erectile dysfunction (Gao et al., 2021). Arginase overexpression has also been linked to immunosuppression in the tumor microenvironment and to the pathogenesis of asthma and certain parasitic infections (Cane et al., 2025). Current pharmacological inhibitors are primarily boronic acid derivatives that mimic the tetrahedral transition state of L-arginine hydrolysis and coordinate the active-site manganese ions (Mortier et al., 2017). Although effective *in vitro*, the clinical translation of these synthetic compounds has been limited by concerns regarding selectivity, off-target effects, and long-term safety. Food-derived peptides offer an attractive approach: they are generated from generally recognized as safe (GRAS) protein sources, can be produced at scale using established enzymatic hydrolysis processes, and are generally associated with a low risk of adverse effects at dietary concentrations.

Research on lupin proteins has remained largely confined to the whole-isolate level, and several gaps persist that this thesis is designed to close. Although *L. albus* and *L. angustifolius* dominate commercial cultivation, it has not been established whether their functional differences arise at the level of the isolate or are concentrated within specific protein fractions; this work demonstrates that functionally and nutritionally superior components such as the sulfur-rich white lupin globulin and the highly digestible blue lupin glutelin are masked when only the isolate is examined. While secondary structure is known to influence protein functionality in general, no predictive structural determinant of lupin emulsification had been identified; this thesis shows that the α -helix-to- β -sheet ratio tracks emulsifying performance and can serve as a marker. Transglutaminase modification of lupin had not been compared across species; this work reveals a species- and pH-dependent response with an identifiable optimal cross-linking window. To date, no study has

systematically screened lupin protein hydrolysates for arginase-inhibitory activity, identified the responsible peptide sequences, or characterized their inhibitory mechanisms. This thesis addresses this knowledge gap by integrating enzymology, chromatographic purification, tandem mass spectrometry, molecular docking, and molecular dynamics simulations.

1.2 Hypotheses

- I. White lupin and blue lupin protein isolates will differ significantly in their physicochemical and structural properties due to species-level variation in conglutin composition.
- II. Fractionation of lupin protein into albumin, globulin, and glutelin fractions, and further separation of the globulins into vicilin and legumin fractions, will yield components with distinct and, in some cases, superior techno-functional properties compared to the unfractionated isolate.
- III. Lupin protein isolates from both species will exhibit emulsifying properties comparable to those of soybean protein isolate, with performance modulated by pH and protein concentration.
- IV. Transglutaminase-catalyzed polymerization of lupin protein isolates will produce high-molecular-weight aggregates with improved emulsifying properties relative to the native proteins.
- V. Enzymatic hydrolysis of lupin protein isolates will liberate peptides capable of inhibiting arginase activity.
- VI. Arginase-inhibitory peptides can be purified by reverse-phase HPLC and their amino acid sequences elucidated using liquid chromatography–tandem mass spectrometry.

1.3 Objectives

The specific objectives of this study are to:

- I. Produce protein isolates from white lupin and blue lupin seeds and characterize their physicochemical, structural, and techno-functional properties.
- II. Fractionate lupin protein isolates into albumin, globulin, and glutelin fractions and evaluate the structure–function relationships of each fraction.
- III. Separate lupin globulins into vicilin (7S) and legumin (11S) fractions and compare their structural and functional properties.
- IV. Compare the emulsifying properties of white lupin, blue lupin, and soybean protein isolates across a range of pH and concentration conditions.
- V. Investigate the effect of transglutaminase-catalyzed cross-linking on the structural and emulsifying properties of white and blue lupin protein isolates.
- VI. Optimize enzymatic hydrolysis of lupin protein isolates to produce hydrolysates with arginase-inhibitory activity.
- VII. Purify arginase-inhibitory peptides from lupin protein hydrolysates, determine their amino acid sequences, and characterize their inhibitory kinetics and binding interactions through molecular docking and molecular dynamics simulations.

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CHAPTER TWO

MANUSCRIPT ONE

LUPIN SEED PROTEINS: A COMPREHENSIVE REVIEW OF COMPOSITION, EXTRACTION TECHNOLOGIES, FOOD FUNCTIONALITY, AND HEALTH BENEFITS

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2.0 ABSTRACT

Plant proteins are rapidly gaining momentum in the drive for sustainable food production. Incorporating these proteins into various food formulations has now been proposed as an alternative to animal proteins because of its numerous benefits on human health, the environment, and sustainable agriculture. Lupins are underutilized legumes and are particularly attractive to the food industry because they have high protein and fiber contents in addition to a low starch content. Unlike most other legumes, lupin contains oil. Despite the proven nutritional and functional benefits, lupins are primarily used as animal feed and do not yet play an important role in human nutrition. For the lupin seeds to become a major player in the food industry, important information on the extraction and purification of proteins and how protein isolates can be exploited in different food formulations is required. This review discusses the underlying principles for dry and wet fractionation of lupin seeds and the influence on yield and techno-functional properties of resultant protein-enriched products. The physicochemical, nutritional, functional, and health benefits of lupin protein isolate, incorporation into different food formulations, and patents on lupin protein technologies were also discussed. This review is timely regarding advancing lupin seed protein research and applications as a sustainable, plant-based protein source for human diets.

Keywords

Lupin proteins, protein isolation, protein functionality, food formulation, health benefits

2.1 INTRODUCTION

The *Lupinus* group is a diverse genus of about 200 species of herbaceous perennials and partly woody plants in the pea family (Fabaceae).^[1] The term lupin, from the Latin word for “Wolf”, derives from the mistaken belief that these plants depleted or wolfed minerals from the soil,^[2] while a more plausible reason might be its uses in the treatment of epilepsy.^[3] On the contrary, the lupin plant is a legume that aids soil fertility by fixing nitrogen from the air into the soil. Plants of the *Lupinus* species, known as lupin (in Australia and Europe) or lupine (in the Americas) are commonly used for ornamental, nutritional, agricultural, and medicinal purposes. Lupins burst into the limelight as a source of plant proteins because they have high protein and non-starch polysaccharide contents than other legume seeds. The protein content of dehulled lupin seeds is in the 39%-53% range (dry matter basis) compared to <25% for soybean.^[4]

Based on geographical distribution, the lupinus genus can be broadly classified into the “old world species” comprising the Mediterranean/North and East Africa, and the “New World species” comprising North and South American species.^[5] Species of the “New World” group are mainly annual and herbaceous perennials and a few shrubs.^[1] Out of all the species of lupin, only four are of agronomic interest: *L. angustifolius* (blue lupin or narrow-leaf lupin), *L. albus* (white lupin), *L. luteus* (yellow lupin), and *L. mutabilis* (Andean lupin).^[6] The wild form of *L. angustifolius* usually has blue flowers and is referred to as blue lupin in Europe. In Australia, all agricultural cultivars of *L. angustifolius* have been bred to have white flowers to distinguish it from its wild bitter relative. Australian growers often refer to them as white lupins (*L. albus* is known as white lupin in Europe). Researchers in Australia often use the name narrow-leafed lupin, while industry refers to it as Australian sweet lupin.

Lupins are historically relevant in food and agricultural practices dating far back as 2000 years ago as the seeds were consumed in ancient Egypt and pre-Inca civilization.^[2] Roman agriculturists deduced the important role played by lupin in soil fertility. Lupins also form an important component of popular diets in the Mediterranean and Andean mountains. Although lupins were a relative latecomer to domestication in the Mediterranean, “Tarwi” was a staple in the Andean diet, when the Pre-Incan population domesticated lupin as early as 2000 BC.^[7] Lupins were introduced into northern Europe from the Mediterranean by Frederick the Great of Prussia to improve the soil fertility of Northern Germany.^[7] By the end of the 18th century, lupin was widespread across the sandy soils of the Baltic coastal plain for forage and green manure and moves were made by German scientists at the turn of the century to domesticate lupin. The goal was to produce a sweet variety of lupin because alkaloids in the undomesticated bitter variety make it undesirable for human and animal consumption. The successful introduction of lupin cultivars with the required "sweet gene" paved the path for lupins to be more widely accepted in Europe and Australia. Western Australia is the world's leading producer of lupins, but the crop is also grown in Eastern Europe, South Africa, South America, Canada, and the United States of America (USA).^[4]

2.2 Lupin Seed Protein Classification

The main proteins of leguminous seeds are usually found in the storage vacuole and are predominantly but not exclusive members of storage proteins that serve as nitrogen and carbon skeleton sources for emerging plantlets.^[8] According to the Osborne classification, legume proteins can be divided into four groups: albumins (water soluble), globulins (salt soluble), prolamins (alcohol soluble), and glutelins (acid/alkali-soluble). Globulins (also known as conglutin) constitute the major fraction of lupin seeds proteins followed by albumins (9:1 globulin to albumin ratio), with prolamins and glutelin making up the remainder.^[8] Lupin seed storage

proteins can also be classified based on the sedimentation coefficient as 2S (commonly referred to as albumin), the 7S and 11S classes (sub-classes of the globulins).^[9]

Blagrove and Gillespie ^[10] using the cellulose acetate electrophoresis technique, separated the proteins from *L. albus* and *L. angustifolius* into four main fractions. These fractions were named α -conglutin (11S), β -conglutin (7S), γ -conglutin (7S) and δ -conglutin (2S) based on their electrophoretic mobility. The subjection of these fractions to further separation techniques involving isoelectric focusing and ion exchange chromatography illustrated the acidic nature of α -, β -, and δ -conglutins and the basic nature of γ -conglutin. The most abundant fraction in *L. albus* and *L. angustifolius* is α and β -conglutin, which make up about 80% of the total proteins in the seeds.^[11] The most abundant conglutin in *L. angustifolius* based on transcript level is β -conglutin (56%), followed by α -conglutin (24%), δ -conglutin (15%) and γ -conglutin (6%).^[12] In *L. albus* the ratios are β -conglutin (45%), followed by α -conglutin (37%), δ -conglutin (12%) and γ -conglutin (5%).^[8]

2.2.1 α -conglutin

L. albus α -conglutin (11S) with a molecular weight of 330-430 kDa belongs to the legumin class of globulins. It exists as a hexamer, however, before conversion to the mature form, α -conglutin prevalently exists as a trimer.^[8] The monomeric unit of α -conglutin is made up of an acidic and basic subunit usually referred to as α and β chains, respectively. α -polypeptides with relative molecular mass (M_r) of 47-52 kDa and 42-44 kDa as well as β -polypeptides of 20-22 kDa have been identified in matured *L. albus* seeds.^[13] Using proteomic analysis, Magni *et al.* ^[14] identified at least two acidic and five basic chains from these polypeptides. The major post-translational modification occurring in α -conglutin is glycosylation, as some of its subunits are glycosylated, which is contrary to most legumin-like globulins.^[8] The acidic subunit's glycosylation consensus

sequence residues were first reported by Duranti and colleagues [15]. The presence of α -conglutin in the storage vacuole suggests that it serves as a storage protein, and the significant proteolytic breakdown that occurs during germination supports this role.[8] However, *in vitro* proteolysis experiments and germination studies show that the basic component, which appears to be in the protein core, is more resistant to proteolytic cleavage than the acidic subunit.[13]

L. angustifolius α -conglutin (11S) also exists as a hexamer with the monomeric unit made up of acidic and basic chains that are derived from the same precursor and linked via disulfide bridges [12]. The three α -conglutin sequences from *L. angustifolius*, which are identified in the Uniprot database are α -1 to α -3 with M_r of 57.8, 74.2 and 67.4 kDa, respectively. Among this sequence, α -1 is more divergent, while α -2 and α -3 have similar structural properties. [16]

2.2.2 β -conglutin

The *L. albus* β -conglutin (7S) also named vicilin-like globulins is the most abundant lupin seed protein.[8] β -conglutin exhibits a trimeric quaternary structure consisting of several monomeric polypeptides with M_r ranging from 16 to over 70 kDa. Duranti and colleagues[17] identified and purified a 64 kDa β -conglutin precursor polypeptide. This polypeptide is glycosylated and contains many proteolytic processing sites which may be responsible for the abundance of 7S mature polypeptides.[17] β -conglutin does not contain disulfide bridges as shown by the identical results from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) bands obtained under reducing and non-reducing conditions.[17] β -conglutin is located within protein bodies in the seed and total degradation during germination supports its role as a storage protein.[8] Rodrigues dos Ramos *et al.*[18] showed that the accumulated 20 kDa breakdown product of β -conglutin catabolism in cotyledons of germinating *L. albus* seeds displayed lectin-like activity. β -conglutin from *L. angustifolius* also displays a trimeric structure devoid of disulfide bridges to link the

monomeric units. The gene exhibits a high degree of similarity to each other with conservation in the hydrophilic domain when compared to 7S globulin sequences from other legume species.^[16]

2.2.3 δ -conglutin

δ -conglutin (2S) belongs to the family of sulfur-rich albumin. δ -conglutin is a monomer comprising of two subunits with a M_r of 4 kDa and 9 kDa. The two subunits are linked via interchain disulfide linkage at Cys^{a8}–Cys^{b24} and Cys^{a20}–Cys^{b13} or, more likely, Cys^{a20}–Cys^{b12}. In addition, the large polypeptide contains two intrachain disulfide bridges (Cys^{b26}–Cys^{b68} and Cys^{b12}–Cys^{b60} or, more likely, Cys^{b13}–Cys^{b60}).^[19] There is insufficient information on the localization and biological function of δ -conglutin. Gayler *et al.*^[20] described the localization of a homologous protein in *L. angustifolius* in the storage vacuole. Findings from this research about the localization may also be inferred for δ -conglutin from *L. albus*. In *L. angustifolius*, four gene sequences of δ -conglutin (δ -1 to δ -4) with M_r of 17.8, 17.8, 17.4 and 10.7 kDa have been identified. It was observed that the δ -1 to δ -3-conglutin sequence share close alignment and similarity while δ -4-conglutin appeared to contain several deletions.^[16] δ -conglutin is rich in cysteine residues and it accounts for roughly 70% of lupin seed sulfur content.^[12]

2.2.4 γ -conglutin

γ -conglutin is a basic 7S protein that is soluble in both water and salt solutions. At neutral pH, the prevalent form is a tetramer or hexamer because of the detected size of the oligomer, which supports both descriptions.^[8] At acidic pH, the oligomer dissociates to a monomer of about 50 kDa, with each monomer consisting of two disulfide-linked subunits of 17 and 29 kDa. Both subunits are heterogeneous and post-translational proteolysis may be the reason why both subunits exhibit heterogeneity.^[8] The 29 kDa chain is glycosylated with covalently linked mannose and glucosamine units.^[21] γ -conglutin is reported to be rich in the amino acid methionine.^[12] γ -

conglutin is located in the protein bodies in germinating seeds.^[22] However, this protein has also been detected in the extracellular apoplastic regions of germinating lupin cotyledon.^[8] Stability to proteolysis by proteolytic enzymes during the germination phase and *in vitro* tests suggest that γ -conglutin is not a storage protein. γ -conglutin binds divalent metal ions such as Zn^{2+} and Ni^{2+} , which may be responsible for promoting refolding of the denatured protein and exhibiting glucose modulatory properties.^[6,23]

2.2.5 Other relevant lupin seed proteins

The most abundant proteins in lupin seeds are α -conglutin (11S), β -conglutin (7S), γ -conglutin (7S) and δ -conglutin (2S) and they make up about 85-89% of the total seed proteins. These proteins are responsible for the major properties of the seeds, flour and derived product behaviour. Some minor proteins have been detected in lupin seeds, and they include the Bowman-Birk serine proteinase inhibitor (BBI) identified by Scarafoni *et al.*^[24] The BBI isolated from lupin seed is peculiar because it is a double-headed trypsin inhibitor rather than the common trypsin/chymotrypsin inhibitor type. The BBI from other plants has shown potential anti-inflammatory and anti-carcinogenic effects and it will be interesting to see what lies ahead for lupin BBI.

Although present in relatively low abundance, several additional lupin proteins contribute disproportionately to the seed's nutritional, functional, and immunological properties. In addition to Bowman-Birk inhibitors (BBIs), lupin seeds contain Kunitz-type serine protease inhibitors. However, modern sweet-lupin cultivars exhibit negligible trypsin-inhibitory activity, considerably lower than that of soybean and most other legumes, thereby eliminating the need for thermal pre-treatment to inactivate these antinutritional factors.^[59] Similarly, lectins are present at very low or undetectable levels in domesticated lupin, representing a further nutritional advantage over many

conventional legumes.^[59] Among the minor allergenic proteins, non-specific lipid transfer proteins (nsLTPs) are small cysteine-rich proteins stabilized by multiple disulfide bonds, making them highly resistant to heat and gastrointestinal digestion. The *L. angustifolius* nsLTP has been identified as an allergen, while pathogenesis-related (PR-10) proteins and profilins constitute additional minor allergenic components.^[59] These proteins complement the major lupin allergen, β -conglutin (Lup an 1), whose structural homology with the peanut allergen Ara h 1 accounts for the well-documented cross-reactivity between lupin and peanut.^[8] In addition, lupin seeds contain oil bodies that store lipids and are stabilized by oleosins, amphipathic structural proteins located at the oil–water interface. Although lupin oleosins remain poorly characterized than those of other oilseeds, they are increasingly recognized for their roles in oil-body stability, natural emulsification, and potential allergenicity.^[59]

Table 2.1 Lupin conglutin's isolation procedures

	Lab scale	Pilot plant isolation process	Scalable isolation process
Raw Material	Flour	Flour, flakes	Flour
Protein extraction	Neutral pH 0.5 M NaCl	Alkaline conditions	Alkaline conditions
Fractionation	Anion exchange chromatography ↓ Con A affinity chromatography ↓ Cation exchange chromatography ↓ Insulin affinity chromatography ↓	Isoelectric precipitation pH 4.0- 5.0 ↓	Isoelectric precipitation pH 4.0- 5.0 ↓ Acidic Water/ethanol Precipitation ↓ Precipitation With Zn^{2+} ↓
Final protein product, conglutins	α δ β γ	α + β + δ γ	α+β δ γ

Modified from Duranti *et al.* [8]

2.3 Dry Fractionation

Dry fractionation is gaining attention for its low energy use, modest particle disclosure and absence of water in the entire procedure. [25] The procedure also does not require drying. Dry fractionation involves a combination of milling and air classification, and the proteins present in the final product still retain their native functional properties.[26] Pulverization of lupin seeds is usually done

by hammer milling, pin milling or jet milling.^[7] Lupin seed proteins are found in protein bodies and dry milling generally detaches starch granules from smaller protein bodies in the flour. Air classification further separates starch granules from protein bodies in the flour based on size and density.^[27] Smaller protein-rich fragments (3 μm) are separated from the larger starch granules (25-40 μm) by airflow. While intensive milling thoroughly detaches the protein bodies from other cellular components, it also has the drawback of milling the fibre component into smaller fragments that accumulate in the protein-rich segments to lower the overall protein content.^[28]

Pelgrom *et al.* ^[26] carried out a study to prepare functional lupin protein fractions by dry fractionation. The lupin seeds were pre-milled into grits using a pin mill (LV 15M, Condux-Werk, Wolfgang Bei Hanau, Germany). The size of the grits obtained was $196.3 \pm 7.5 \mu\text{m}$ and it was further milled using a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany). The airflow was set at 80 M^3/H and the classifier wheel was set at 1000, 2500, 4000, or 6000 rpm. The batch size of 1 kg was processed at a time, while the impact mill speed was 8000 rpm and at a feed rate of 2 rpm. The milling yield was estimated as the total mass of the milled flour versus the mass of the lupin grits. The highest protein content obtained was 53 g/100 g dry matter with a protein yield of 12.8 g/100 g at 1000 rpm classifier speed. This was further air classified at an increased classifier wheel speed with 500 g of flour for each batch at a feed rate of 15 rpm. The flour was air classified using an ATP50 classifier (Hosokawa-Alpine, Augsburg, Germany). The airflow was set at 80 M^3/H and the classifier wheel at 7000, 10,000, or 13,000 rpm. This led to a significant increase in the protein content (58.9 g/100 g dry matter). The yield of the flour decreased as the classifier wheel speed increased due to fouling (material loss) on the mill's inner walls, which may be attributed to the smaller particle size and greater exposure of the lipids.^[26]

Flowability aid has been proposed to enhance the air classification of lupin seed flour.^[26] Flowability aid increases the distance between particles, reducing the attraction forces and hence clump formation. Aerosil and potato starch are the common food-grade flowability aids employed in the industry.^[29] The addition of Aerosil and potato starch as flowability aids in lupin flour during air classification resulted in a slight decrease in the protein concentration to approximately 53.9 ± 1.8 g /100g dry matter, which is less than 59 ± 0.0 g/100g protein content obtained without the flowability aid.^[26] Potato starch further decreased the yield of the fine flour and the authors postulated that the proteins may have become attached to the starch granules, making them bigger and separating in the coarse fraction. Thus, in this study, the presence of dispersibility aid did not improve separation because the protein bodies and fibres are of similar size.^[26]

Wang *et al.*^[28] investigated the potential of applying electrostatic separation based on tribo-electrostatic charge and subsequent separation in an electric field to further enrich the protein in lupin flour. Protein and fibre become positively or negatively charged while sliding down a surface due to their different tribo-electric characteristics. The dosing rate of lupin flour subjected to electrostatic separation is 1.25 kg/hr, an applied voltage of 20kV, a gas flow rate of 10 L/min and a distance of 10 cm between the electrodes. Lupin protein was shown by Pelgrom *et al.*^[27] to take up a positive charge and subsequently deflected towards the ground electrode to produce protein-enriched fractions. After three rounds of electrostatic separation of the flour milled at 2500 rpm, the protein content was found to increase from 51.4 to 65.0 g/100 g solid, which is approximately a 15% increase than obtained with air classification.^[28]

2.4 Wet Fractionation

The wet fractionation process aims at producing protein isolates with high purity to better understand how each protein fraction might be useful as a potential ingredient in the food sector. However, the process consumes water, energy, and solvent. ^[25] Large-scale wet fractionation processes generally involve oil extractors, sample mixers, decanters, solid separators, and spray driers. Oil extraction and spray drying are the most energy-intensive procedures while mixing and decanting require less energy. ^[25] Wet fractionation enriches lupin protein fractions in the form of concentrate and isolates. This method also lowers the alkaloid and other antinutritional factors contained in the samples. Wet fractionation is broadly divided into conventional wet fractionation and aqueous fractionation. Conventional wet fractionation employs the use of alkaline and acidic solutions to exploit the inherent solubility properties of proteins during extraction. The three major isolation techniques involved in wet fractionation are (i) alkaline extraction followed by ultrafiltration or isoelectric precipitation (ii) micellization (salt-based solubilization) followed by dilutive precipitation (iii) selective fractionation (acid extraction). The isolation technique employed depends on the cost and techno-functional properties desired.

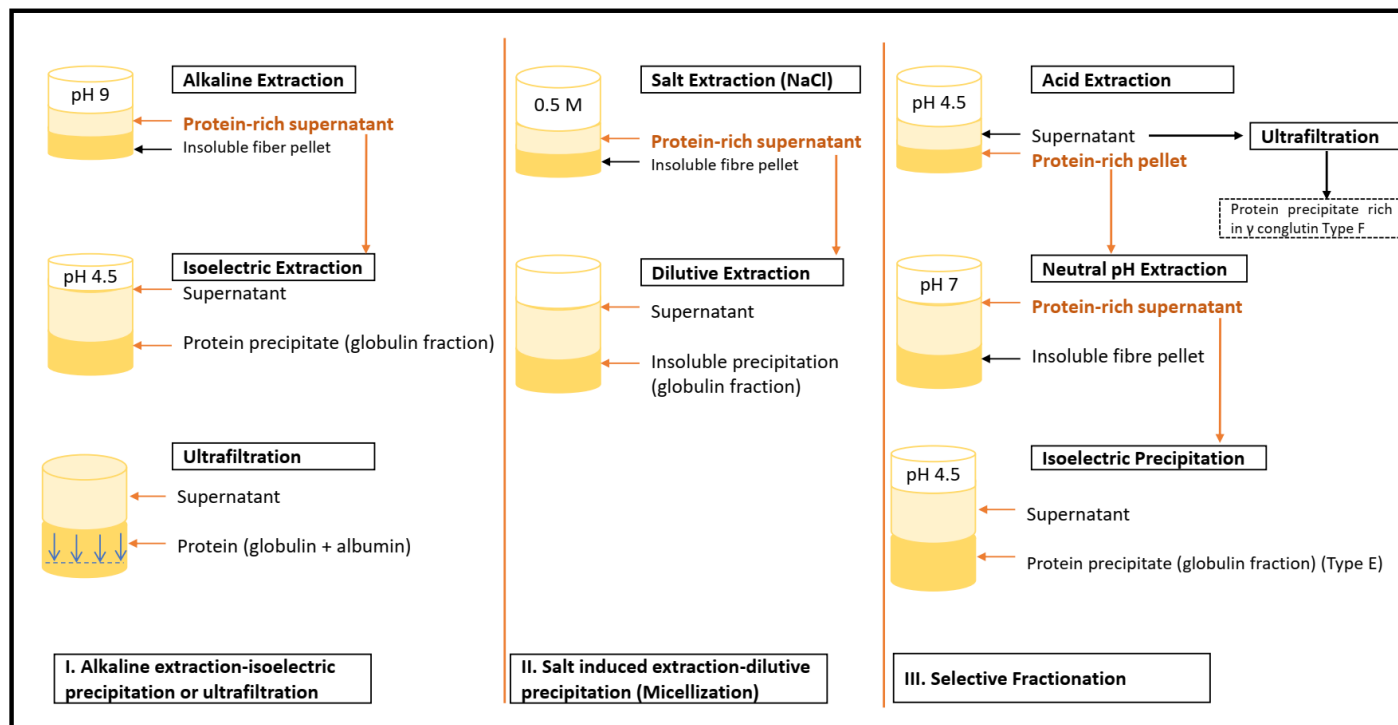


Figure 2.1 Techniques for lupin protein isolation. Source: modified from Shresta et al. [12]

2.4.1 Alkaline extraction

Protein charge and solubility are exploited in this technique for lupin protein isolation. At alkaline pH values, negatively charged proteins tend to display repulsions that favor protein solubilization, while at the isoelectric point, proteins become less soluble because the net charge is zero. [12] Ultrafiltration can also be employed to concentrate and purify proteins with minimal protein degradation. Lupin flour or flakes is the starting material. Full or defatted lupin flour or flakes are dissolved in water (1:8 w/v), the pH adjusted to alkaline condition (pH 8-12), the sample is stirred for 1-2 h at room temperature to allow protein solubilization and then centrifuged to separate the protein extract from insoluble materials. To achieve protein precipitation, the soluble protein

extract is adjusted to pH 4.5 with an acid solution and left to stand for 8-24 hours at 0-4 °C. Centrifugation is carried out next to recover the precipitated lupin proteins, which are finally washed with distilled water (ratio 1:10) to remove excess salt. The alkaline extraction process can be combined with membrane ultrafiltration and diafiltration, which are effective in extracting globulins and albumins. [30] Studies carried out by Jayasena *et al.* [31] showed that there was no significant difference in the protein content and yield of lupin protein isolates precipitated at pH 4.4, 4.5, 4.6, 4.8 or 5, which covers the isoelectric point of most leguminous proteins. Precipitation of lupin proteins can thus be carried out at a level such as pH 5 in an economical approach aimed at reducing the wastage of acids.

Dried lupin protein isolate powders are produced via freeze-, drum-, spray-, or vacuum drying. Drying is an energy-intensive step and the method employed also has a distinct effect on the physicochemical and techno-functional properties of protein isolates and concentrates. [27] For samples that are not defatted, alkaline extraction results in products with the highest fat content. [30] This is because, during protein solubilization, the fat components can become saponified to form emulsions in the presence of NaOH, which become incorporated into the protein structure thereby increasing the overall fat content of the isolated proteins. [30]

2.4.2 Micellization (salt extraction)

Micellization followed by dilutive precipitation is a mild process, when compared to alkaline extraction coupled with isoelectric precipitation because it avoids the use of extreme pH that may lead to protein denaturation. [32] The extraction and precipitation stages play a decisive role in microstructure orientation during micellization. The lupin flour or flake is dissolved in 0.5 M NaCl at a ratio of 1:8 (w/v). This is followed by continuous stirring for 1 h at room temperature. Sieving of the suspension is carried out next to remove the fiber component followed by centrifugation at

3000 g for 10 min. Filtration of the supernatant to remove fine solids from the extract yields clear supernatant. To precipitate proteins from the extract, the supernatant is diluted with deionized water at a ratio of 1:3 (v/v) at room temperature with constant stirring, before allowing to stand at 1 °C for 18 h. The salt-soluble proteins lose solubility as a result of the low ionic strength of the solution, which leads to the formation of a precipitate that is recovered by centrifugation and further washed (1:10 (w/v) with deionized water. [30] The protein isolates obtained can be stored at -20 °C or -80 °C prior to lyophilization. The protein content of isolated proteins obtained from alkaline extraction and salt extraction usually range from 90%-96% based on dry matter. [32]

2.4.3 Selective Fractionation (Acid Extraction)

Lupin seed flour or flake treated with an acidic solution lead to the separation of low molecular weight substances such as oligosaccharides, alkaloids, and γ -conglutin fraction from high molecular weight α -, β - and δ -conglutin and fibres. This extraction is carried out at pH 4.5 at 12 °C for 45 min. Concentration of the clarified acid extract via ultrafiltration or diafiltration yields the lupin protein isolates labeled as type F (γ -conglutin) as shown in Figure 2.1. This selective fractionation process leads to the recovery of lupin's γ -conglutin, which is usually lost during the alkaline extraction procedure. [4] The protein-rich pellet is further subjected to neutral extraction to separate high molecular weight proteins from the residual dietary fiber. This is carried out at pH 7 and 30 °C for 30 min followed by centrifugation. The resulting extract is precipitated at the isoelectric condition (pH 4.5 using HCl for 10 min) to obtain a protein isolate designated as type E. [4]

2.5 Overview of lupin protein extraction

Several studies have reported on the challenges encountered in the isolation of lupin proteins. In a study by Albe-Slabi et al. [33], it was reported that the extraction of lupin proteins from various cultivars using different methods such as acid and alkaline extraction resulted in varying protein yields and qualities. The extraction pH appears to have the greatest influence on quality of the lupin isolates, whereas the purification process does not seem to have a very significant impact on the yield and characteristics of the protein. Upon further characterization, the authors noted that the lupin proteins extracted at pH 2 (acid extraction) exhibited partial denaturation, which led to loss of protein solubility, modification of secondary structure, low thermal stability, and formation of aggregates. Removing anti-nutritional factors, such as alkaloids and protease inhibitors during the extraction stage is beneficial because they can interfere with protein extraction and purification. [4] Generally, the use of harsh extraction conditions, such as high temperature and alkaline pH, can result in protein denaturation and aggregation, which can affect protein solubility and functionality. [4] Similarly, in a study by Devkota et al. [34], the authors reported that the isolation of lupin protein fractions from *L. albus* and *L. angustifolious* using alkaline extraction and isoelectric pH precipitation coupled with freeze drying, spray drying, or pasteurization resulted in varying protein profiles and functional properties. The authors noted that commercial processing conditions do not have an immense effect on the structural properties of lupin protein isolates, which were mainly due to varietal differences.

Another challenge encountered in the isolation of lupin proteins is the presence of allergenic proteins. In a study by Schlegel et al. [35], the authors reported that lupin seeds contain several proteins that can trigger allergic reactions in susceptible individuals. The authors noted that a combination of enzymatic hydrolysis and fermentation of LPI was effective in reducing the major

allergen (Lup an 1) to a residual level of <0.5%. The combination of enzymatic hydrolysis and lactic acid fermentation is effective for the removal of allergenic proteins during protein isolation and purification and is critical to ensure the safety of lupin-derived food products. The influence of different extraction buffer systems on the proteome of blue lupin seeds was investigated by Tahmasian et al. [36], and observed that there is a distinct difference in the proteome of Tris-HCl and urea-extracted cultivars. The authors concluded that the extraction buffer utilized had an impact on proteome coverage, downstream functional annotation results, and hence biological interpretation, emphasizing the need to optimize and understand the impact of protein extraction conditions. Moreover, the genetic variability of lupin cultivars can also affect protein isolation. In a study by Cabello et al. [9], the protein content and composition of lupin seeds varied depending on the cultivar. Proteomic analysis showed that lupin seed protein diversity goes beyond gene diversity because the level of protein is correlated with differential proteolytic cleavage of conglutin pro-proteins and post-translational glycosylation.

The extraction solvent for lipids found in lupin seeds can influence the yield and recovery of lupin proteins. Bader et al. [37] examined the yield and recovery of lupin protein after using a wide range of organic solvents for lipid extraction. According to the study, 36-44% of the protein was recovered. The highest recovery of 44% yield was obtained by using diethyl-ether ethanol to extract fat before protein extraction, whereas the lowest yield of 36% was obtained when ethanol was used. De-oiling with ethanol led to increased protein denaturation resulting in decreased solubility and thus lower protein recovery. The solubility of lupin protein is a driver for many physical functionalities. It is influenced by the protein extraction method, temperature, and drying method. Alkaline extraction and isoelectric precipitation usually yield lupin protein with a solubility of approximately 92-98%. [8] Solubility of lupin protein is highest at pH 8-10 and lowest

at the isoelectric point (pH 4.5). Berghout et al. [38] compared the solubility of lupin protein extracted via alkaline extraction and isoelectric precipitation at 4 °C and 90 °C. The solubility at 4 °C was 90 % when compared to 76% at 90 °C. This decrease in solubility may be a result of the thermal denaturation of the protein, therefore, the result demonstrated that thermal conditions also influence the solubility of extracted lupin proteins. In a study by Pozani et al [39] the effect of the addition of dithiothreitol (DTT) and temperature on solubility of acid-extracted lupin protein isolates was investigated. The protein solubility was reduced from 80% to 20% when heated to 80 °C for 15 min. In contrast, the protein solubility was unaffected by the addition of DTT only. However, the solubility was reduced from 80% to 70% after adding DTT and heating to 80 °C.

2.6 Nutritional Properties of Lupin seed protein

Comparative studies carried out by Vogelsang-O'Dwyer *et al.* [4] showed that *Lupinus albus* protein isolate (WLPI) and *Lupinus angustifolius* protein isolate (BLPI) have comparable protein content at >90 % on dry weight basis as shown in Table 2.2. The fibre content in both isolates was <0.1 % indicating that fibre was eliminated during the isolation stage. The mineral content of protein isolates from both species is comparable except for Iron which was significantly higher in BLPI than WLPI and manganese which was significantly higher in WLPI than BLPI. The sodium content is significantly higher in both protein isolates, which may be due to the sodium hydroxide used in neutralization before drying. The presence of high content of sodium hydroxide can impact the degree of suitable applications for the protein isolates.

The limiting amino acid in *L. albus* and *L. angustifolius* protein isolates is the sulfur-containing amino acids methionine and cysteine, which is commonly deficient in legume seeds. [7] The data from Vogelsang-O'Dwyer *et al.* [4] shown in Table 2.3 indicate that lysine and valine are also limiting in both WLPI and BLPI. Tryptophan was also found to be limited in WLPI. The

combination of phenylalanine and tryptophan in WLPI was higher when compared with BLPI. The overall amino acid profile of lupin protein isolates may be improved if it is complemented with proteins from cereals that are known to be rich in sulfur-containing amino acids.^[40]

Table 2.2 Protein content and mineral composition of *Lupinus albus* and *Lupinus angustifolius* protein isolates

	BLPI	WLPI
Macronutrients (g/100 g)		
Protein*	92.8 (84.5)	94.4 (86.1)
Fat	0.92	1.07
SFA	0.48	0.65
MUFA	0.25	0.43
PUFA	0.2	<0.10
TFA	<0.10	<0.10
ASH	5.3	4.4
Moisture	5.52	3.81
Dietary fiber	<0.1	<0.1
Minerals (mg/kg)		
Sodium	14,600	11,740
Potassium	290	320
Phosphorus	9400	8200
Iron	110	60
Zinc	25	22
Copper	11	8
Calcium	1400	1300
Iodine	<10	<10
Magnesium	210	220
Chromium	<1	<1
Manganese	17	170
Molybdenum	2	1
Selenium	1	1
Chlorine	<5	<5

Protein content is shown as N×6.25 and as N×5.7 in parentheses for comparison. SFA: saturated fatty acids; MUFA: mono-unsaturated fatty acids; PUFA: polyunsaturated fatty acids; TFA: trans fatty acids.

Source: Modified from Vogelsang-O'Dwyer *et al.* [4]

Table 2.3 Amino acid profile of *Lupinus albus* and *Lupinus angustifolius* protein isolates

	BLPI		WLPI	
	Level (g/100g protein)	% of Requirement	Level (g/100g protein)	% of Requirement
Indispensable/conditionally indispensable amino acids				
Histidine	2.53 ± 0.31	168	2.09 ± 0.25	139
Isoleucine	3.97 ± 0.48	132	4.49 ± 0.54	150
Leucine	6.86 ± 0.83	116	7.62 ± 0.92	129
Lysine	4.19 ± 0.51	93	4.25 ± 0.52	95
Methionine	0.33 ± 0.02		0.26 ± 0.02	
Cysteine	1.13 ± 0.08		1.10 ± 0.08	
Methionine + cysteine	1.46 ± 0.11	66	1.36 ± 0.10	62
Phenylalanine	3.88 ± 0.47		4.60 ± 0.56	
Tyrosine	3.01 ± 0.37		5.23 ± 0.63	
Phenylalanine + tyrosine	6.90 ± 0.84	182	9.83 ± 1.19	259
Threonine	3.29 ± 0.4	143	3.30 ± 0.4	144
Tryptophan	0.67 ± 0.1	112	0.57 ± 0.1	95
Valine	3.46 ± 0.42	89	3.65 ± 0.44	94
Dispensable amino acids				
Aspartic acid	9.59 ± 1.16		10.81 ± 1.31	
Glutamic acid	22.77 ± 2.76		23.13 ± 2.8	
Alanine	2.93 ± 0.36		2.94 ± 0.36	
Arginine	11.71 ± 1.42		11.60 ± 1.41	
Glycine	3.86 ± 0.47		3.79 ± 0.46	
Proline	4.41 ± 0.53		3.97 ± 0.48	
Serine	4.88 ± 0.59		5.57 ± 0.67	

Source: Modified from Vogelsang-O'Dwyer *et al.* [4]

2.7 Functional properties of lupin seeds

2.7.1 Solubility

Protein solubility is a significant attribute for many food and beverage applications with relevance to various functional properties like gelation, emulsification and foaming. In line with earlier findings for high protein components based on lupin and other legumes, Vogelsang-O'Dwyer et al. [4] found that protein solubility dropped as acidity neared pH 4.5-5.0, the isoelectric point (pI). At this pI, minimum solubility (8–10%) was observed. The zeta potential findings showed that white lupin protein isolate (WLPI; *Lupinus albus*) and blue lupin protein isolate (BLPI; *Lupinus angustifolius*) have a zeta-potential of 0 mV around pH 4.5. One distinguishing feature was that WLPI was more soluble at pH 3.5, which may be advantageous in some applications involving acidic foods or beverages. For both lupin protein isolates, the influence of pH on solubility was low between pH 6.5 and 8.0. At pH 7, the solubility of WLPI was 69.8% and BLPI was 76.9%. In a study carried out by Schlegel *et al.* [41], protein solubility at pH 4 after enzymatic and fermentation treatment of LPI was significantly different from the untreated protein isolates. With values ranging from 19.7 to 36.7%, all fermented lupin protein hydrolysates demonstrated significantly improved protein solubility. After fermentation of the papain hydrolysate S2, the lowest protein solubility (19.7%) was found with *Lactobacillus helveticus*. When compared to solubility of LPI at pH 8.0, single hydrolysis of LPI with Alcalase 2.4 L resulted in a significant improvement in protein solubility at 84.8% as against 79.5% for LPI.

2.7.2 Water holding capacity

The measure of the amount of powdered substance that can be dissolved in water until the saturation point is reached is known as the water holding capacity (WHC) or water absorption capacity (WAC). [42] WHC is important when formulating certain foods because it affects the

texture, flavor retention, mouthfeel, and shelf life of the food product. ^[43] The inability of a protein to bind water could result in the formulation of stiff and dry products. Proteins must gel or solidify at a WHC of roughly 3 mL/g protein to generate semi-solid high-protein diets. ^[38] Remarkably, the protein isolate generated at 90 °C has a WHC that is within the acceptable range for these high-protein diets. However, during fractionation at 90 °C, Berghout and colleagues^[38] discovered that off-odors and color changes were detected, indicating that the protein isolates were undergoing unwanted chemical reactions. They also discovered that combining a post-process heating step at 90 °C with a protein isolate produced at 4 °C resulted in an increase in WHC, which looks like a good alternative because this approach did not produce any off-odors.

2.7.3 Emulsion formation and stability

The amphiphilic nature of proteins has been linked to the formation of emulsions and foams. At the oil-water and air-water interfaces, an amphiphilic molecule can be adsorbed, which lowers surface tension values and encourages the development of emulsions and foams. ^[44] During alkaline aqueous extraction, most of the lipid components are not extracted, however, in the presence of NaOH, the lipid component undergoes saponification to form an emulsion. ^[41] The steady stirring of the flour-NaOH slurry in the extraction step may possibly lead to saponification of the lipid component. Consequently, these emulsions may have emulsified more lipid into the aqueous phase, which contributes to the increased total lipid content of the protein isolates. For example, Bader et al. ^[45] looked at the emulsification properties of proteins extracted from two important lupin types, *L. angustifolius*, and *L. albus*, as well as the effect of homogenization on their emulsification activities. The emulsification properties of *L. angustifolius* (780 mL oil/ g) were found to be superior to those of *L. albus* (580 oil/ g). At 50–100 MPa, there was no effect on emulsification capacity (EC) after homogenization. However, at a greater pressure of 150 MPa in

high-pressure treatments, *L. angustifolius* proteins displayed a higher proclivity for emulsification (780 oil/ g).

Burgos-Daz *et al.* [46] studied the creaming behaviors of lupin emulsified at various oil concentrations and found that creaming properties are influenced by a variety of parameters such as thermal treatment, oil content, and lupin protein particle aggregation. The emulsions were also shown to be highly stable against creaming after being stored for 14 days. Schlegel *et al.* [41] investigated the emulsion properties of untreated and enzyme-treated LPI. All the enzymatically treated LPI displayed significantly lower emulsifying properties when compared to the untreated LPI (620 mL/ g) except for protease N-01 (679 mL/g), corolase N (653 mL/g), and pepsin (623 mL/g). There is a strong correlation between EC and solubility, more protein will be present in the oil phase and continuous phase during emulsification if the protein is readily soluble. Highly soluble isolates like corolase N (78.4% solubility at pH7) have a higher EC (653 mL/g), compared to less soluble flavourzyme 1000L hydrolysates (46.9% solubility at pH 7) with EC of 300mL/g. [41]

Mayonnaise is another example of an oil-in-water emulsion. The major components of mayonnaise are water, egg yolk, oil, salt, sugar, sweetener, and other optional ingredients. [47] The proteins in the mixture act as a stabilizer, textural modifier, and microstructure determinant of the emulsion. [47] The replacement of egg yolk with isolated plant proteins is gaining attention in the drive to find alternative protein sources and produce vegan mayonnaise. The goal is to formulate a product with improved sensory properties such as spreadability, mouth-coating, firmness, and surface shine. Another important factor to also consider is the shelf life of the product. Alu'datt *et al.* [48] prepared mayonnaise from proteins extracted from chickpea, broad bean, and lupin flour. The total phenolics of the mayonnaise from lupin flour was 17.52 mg/mL, while the total antioxidant activity

of the mayonnaise prepared from broad bean and chickpea was 88.2 and 86.3% respectively. The formulated mayonnaise could be recommended as a result of its superior value of lightness, antioxidant potential, and functional properties. [48]

Emulsion-based meat products play a significant role in the modern food industry. Meat batters are oil-in-water emulsions made up of protein-coated fat globules (oil droplets) dispersed in a myofibrillar protein gel matrix. [49] Although protein content and fat type were found to have a significant impact on the stability of meat emulsions, [50] the thickness of the interfacial protein film, the integrity, and density of the surrounding emulsion matrix, and the ability of the matrix to maintain its integrity during thermal processing are all important factors to take into consideration. Through hydrophobic association as well as hydrogen bonding, the gelling ability of myofibrillar proteins was improved when microbial transglutaminase was added to pH-treated soy protein isolates (SPI). [51] The addition of microbial transglutaminase to pea protein resulted in producing a robust meat gel by increasing the protein cross-linking. [52]

2.7.4 Gel formation

Protein gelling ability is a crucial feature for many food items. Many food products are formulated in a gelatinous form, which necessitates the use of gelling substances to acquire the necessary texture and consistency. In the past, these applications have depended on proteins from eggs, dairy products, or soy; however, interest in using different legume proteins for this purpose is growing. To lower system energy, globular proteins that have undergone thermal gelation become unfolded as a result of heating, aggregate as a result of newly exposed groups, and form a continuous protein network that gives the structure a more elastic property. For BLPI, the minimum protein concentration for gelation was determined to be 23% and for WLPI, it was found to be 7%. This

behavior could be explained by the enhanced interactions of protein particles as a result of thermally induced swelling. [4]

According to studies carried out by Berghout *et al* [25], LPI and soy protein isolate (SPI) gels have varying consistencies based on visual inspection and manual deformation. After gelation, the LPI gel was easily deformed and did not reform (i.e., no elastic behavior). The SPI gel was a self-standing gel that broke or ruptured when deformed. For the SPI gel, the concentration at which the dispersions stopped flowing was 15% (w/v) and for the LPI gel, it was 18% (w/v). Because the powder was not fully hydrated at this moisture content, dispersing 30% (w/v) SPI in water was impossible. At 30% (w/v), LPI was easily diffused and hydrated. Also at similar protein concentrations, SPI dispersions had a substantially higher viscosity than LPI dispersions. Shear-thinning was observed in both the LPI and SPI dispersions, although the SPI dispersions had a higher shear-thinning behavior than the LPI dispersions. The viscosity at 30% (w/v) LPI dispersion was similar to that of the 12% (w/v) SPI dispersion.

In terms of behavior, SPI gels were not frequency dependent. When compared to strong gels, the behavior of weak gels is more dependent on the frequency at which they are observed. The strong modulus of SPI was higher than that of LPI at all protein concentrations. In contrast to its deformability, LPI gel at high protein content (30% w/v) had a strong modulus that was comparable to that of 24% (w/v) SPI gel. Under the same experimental settings, Berghout *et al.* [25] found that LPI resulted in weaker, less elastic gels than SPI.

2.7.5 Foaming capacity

Both the BLPI and WLPI showed very comparable behaviors concerning foaming capability, with this property improving with increasing protein concentration. [4] Both LPIs demonstrated a foaming capacity of >70% at 3.0 and 3.3% protein contents, respectively. At protein concentrations

greater than 1%, foam stability was close to 90% for both LPIs after 1 hour, with BLPI achieving marginally but significantly higher values at 3.0 and 3.3% protein. Both LPIs demonstrated comparable foaming capabilities, ^[4] suggesting that BLPI's greater surface hydrophobicity did not have a significant influence on foaming behavior. These results suggest that BLPI and WLPI have significant foaming capabilities at neutral pH and could be used in applications requiring foam formation, such as non-dairy desserts and certain bakery applications. A brief techno-functional application of LPI can be found in Table 2.4.

Table 2.4 Techno-functional application of lupin protein isolates

Functional properties of lupin	Application
Solubility	Beverages (high sol.), sports bars (low sol.), meat analogs. [53-55]
Oil holding capacity	Meat analogs ^[56]
Water holding capacity	Meat analogs, yogurt, and cheese ^[57]
Foaming properties	Meringues, whipped toppings, and baked goods ^[4, 58]
Emulsification	Salad dressing, sauces, encapsulation ^[4, 59]
Gelation	Meat analogs, yogurt, tofu, encapsulation ^[60-61]
Nutritional properties	Integrated food-based products ^[7, 62]

2.8 Health benefits

Lupin seeds are emerging as a game changer due to the growing awareness of their health benefits and potential as new food ingredients. Proteins, phenolics, and other secondary metabolites confer on lupin numerous health benefits. Proteins from lupin seeds have been found to possess anti-diabetic, anti-inflammatory, antioxidant, anti-dyslipidemia, and antihypertensive properties.^[63] Bettzieche *et al.*^[64] investigated the hypocholesterolemic activities of a protein isolate from blue lupin and found a more effective lowering of LDL cholesterol by 37% compared to casein. It is believed that the protein isolates stimulate activity of the LDL receptor, which increased the absorption of circulating LDL from plasma into cells. Other studies on the hypocholesterolemic activities of raw lupin flour, cooked lupin flour and LPIs on hamsters and rats also showed significant cholesterol-lowering properties.^[63] Using pig as an animal model with a lipid metabolism similar to humans, Radtke *et al.*^[65] studied the effect of lupin protein isolates compared to casein on the regulation of sterol excretion and messenger RNA expression of intestinal sterol transporter. The plasma levels of cholesterol, LDL, and HDL were lower in LPI-fed pigs than in casein-fed pigs. According to fecal analyses, pigs fed LPI produced more cholesterol in the feces than those given casein. Lower levels of intestinal sterol transporter mRNA were found in lupin-fed pigs compared to casein-fed pigs, suggesting that lupin protein isolate may have inhibited cholesterol absorption in pigs. The cholesterol-lowering effect of LPI, according to Radtke *et al.*^[65] is caused by an increase in fecal cholesterol level and a decrease in intestinal cholesterol uptake.

The glucose-modulating activity of lupin seed is attributed to the γ -conglutin content.^[6] γ -conglutin is involved in the activation of intracellular kinases and adaptor proteins that regulate insulin signaling and metabolism. Lupin protein hydrolysates also act as an insulintropic agents by

directly stimulating insulin secretion from pancreatic beta cells. Insulin resistance (IR) is a major contributor to the development of type 2 diabetes mellitus complications. In this regard, γ -conglutin proteins can reverse IR through a variety of pleiotropic effects, including (a) boosting glucose cellular absorption; (b) regulating critical genes involved in the insulin-signaling system; (c) activation of the IRS-1/PI-3-kinase pathway through the phosphorylation of upstream modulators, such as IRS-1, as well as downstream mediators and (d) improving the overall cellular sensitivity towards insulin. Studies carried out by Tapadia *et al.* [6] showed that lupin γ -conglutin operates as an insulin-mimetic with the peptides obtained by treating the conglutin with pepsin and pancreatin-inducing phosphorylation and activation of protein kinase B. The peptide also activated several of its downstream targets, which may be responsible for metabolic processes such as glycogen synthesis, protein synthesis, and glucose transport. The conglutin-derived peptides suppressed the activity of dipeptidyl peptidase IV, an enzyme that plays a role in reducing the half-life of the incretin hormone. Dove *et al.* [66] conducted an acute post-prandial study on type 2 diabetes patients who consumed three beverages containing 50 g glucose (as control), 50 g glucose plus *L. angustifolius* fibre, and proteins, or 50 g glucose plus soy fibre and protein. Lupin consumption decreased the glycaemic response, though less effectively than soy, but lupin significantly improved the insulin response when compared to the control and soy. A study was carried out by Ward *et al.* [67] to investigate the impact that consistent consumption of foods containing lupin has on glycemic control and blood pressure in those who are afflicted with type 2 diabetes mellitus. After eight weeks of consumption, the study's findings demonstrated that lupin-enriched foods did not have any effect on either the mean blood glucose levels or the post-prandial blood glucose levels of the participants. During the treatment, there was no discernible change in either the systolic or diastolic blood pressure, the heart rate, or the subject's overall body

weight. Ward *et al.* [67] concluded that the intake of lupin-enriched meals regularly had no impact on the glycemic management or blood pressure of people with type 2 diabetes.

Lima-Cabello *et al.* [68] purified recombinant β -conglutin proteins from *L. angustifolius* using affinity chromatography. The isoforms of β -conglutin ($\beta 1$, $\beta 3$, and $\beta 6$) inhibited nitric oxide production and the expression of inducible nitric oxide synthase. The isoforms also inhibited cytokines and chemokines, suggesting potency as anti-inflammatory agents. Lima-Cabello *et al.* [69] purified γ -conglutin from *L. angustifolius* using affinity chromatography to evaluate its anti-inflammatory potential at the molecular level using both a bacterial lipopolysaccharide-induced inflammation and an insulin-resistant pancreatic cell model. γ -conglutin ameliorated the oxidative stress induced by inflammation through lowering inducible nitric oxide synthase transcription level, carbonylation, nitric oxide synthesis, and increasing the level of glutathione as well as positively modulating the enzymatic activities of catalase and superoxide dismutase. γ -conglutin also induced up-regulated transcriptomic and protein expression levels of the insulin signaling pathway, which led to enhanced glucose uptake while decreasing the levels of pro-inflammatory mediators. [69]

2.9 Protein hydrolysates and bioactive properties

Lupin protein isolates can be treated with proteases to yield protein hydrolysates. Some peptide fractions in the hydrolysates may exhibit biological activities, thus referred to as bioactive peptides. Bioactive peptides (with amino acids ranging from 2-20) perform diverse biological functions and inhibit protein-protein interactions due to their small size and specificity. Lammi *et al.* [70] showed that tryptic and peptic peptides from lupin protein inhibited 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in addition to up-regulating LDL receptor and sterol regulatory element-binding proteins 2 levels, which led to increased LDL-uptake in HepG2 cells.

Furthermore, the same peptides suppressed the activity of angiotensin-converting enzyme (ACE), which suggest a potential *in vivo* hypotensive effect.^[71] Lammi *et al.* ^[72] applied a multidisciplinary approach to investigate the bioavailability and activity of lupin peptides. The purpose of the research was to see if these lupin peptides were absorbed by human intestinal Caco-2 cells. Transport tests were done by incubating each lupin peptide combination from the apical side after the cells had been differentiated for 15 days. Basolateral solutions were collected after 4 h and evaluated using HPLC-Chip-MS/MS. The basolateral samples contained eleven tryptic and eight peptic peptides derived from lupin proteins. *In vitro* testing revealed that basolateral peptides retain their ability to block 3-hydroxy3-methylglutaryl coenzyme A reductase activity, and *in silico* docking simulations allowed researchers to speculate which peptides would bind to the active site more efficiently.

Schlegel *et al.* ^[41] studied the effect of hydrolyzing LPI from *L. angustifolious* by nine different proteases to elucidate their techno-functional properties and sensory properties. SDS-PAGE analysis revealed that alcalase 2.4 L, papain, and pepsin were the most effective in degrading the α - and β -conglutin. Most proteolytic enzymes increased the techno-functional properties of LPI, including solubility, emulsifying, and foaming activities. Pepsin hydrolysis had the most impressive effect, which resulted in an increased foaming activity from 980% in LPI to 3,614% in hydrolysate. The alcalase hydrolysate showed a significant increase in bitterness, which was most likely due to the release of bitter peptides, but the other hydrolysates did not exhibit any significant increase in bitterness intensity when compared to the LPI. Other sensory characteristics of the hydrolysates were similar to those of LPI, except for alcalase treatment. The influence of enzymatic hydrolysis on the solubility and emulsification properties of lupin proteins was investigated by Opazo-Navarrete *et al.* ^[73]. According to the study, neutral and alkaline proteases

were more efficient in hydrolyzing lupin proteins. All the lupin protein hydrolysates with the exception of the acidic protease-treated hydrolysate displayed a higher emulsifying capacity than the undigested lupin protein hydrolysate. The authors noted that solubility and emulsification properties of lupin proteins can be improved by enzymatic hydrolysis.

Cruz-Chamorro *et al.* [74] investigated the immunological and oxidative responses of non-immortalized human cells after being exposed to lupin protein hydrolysates. The goal of this study was to determine how lupin protein hydrolysates from *L. angustifolius* affected the immunological and oxidative conditions of human peripheral blood mononuclear cells (PBMCs) from 53 healthy donors when administered *in vitro*. Lupin protein hydrolysates suppressed PBMC proliferation and levels of pro-inflammatory cytokines Th1, Th9, and Th17 without being cytotoxic. In addition to shifting the pro-inflammatory/anti-inflammatory balance in favor of a Th2-protective response, lupin protein hydrolysates also boosted the activities of superoxide dismutase and catalase, as well as overall antioxidant capacity. Guo *et al.* [75] investigated the protective properties of LPI against oxidative stress. The hydrolysates were generated from the treatment of lupin protein isolate with alcalase, trypsin, and pepsin. When compared to lupin proteins, all the hydrolysates had improved antioxidant and ACE-inhibitory properties. The hydrolysates were divided into three fractions based on molecular weight, with the MW 3 kDa peptides (LPH3) having the strongest antioxidant and ACE-inhibitory properties. In HepG2 cells, the LPH3 fraction provided the best protection against reactive oxygen species formation, which was linked to enhanced superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities via overexpression of SOD1, GPX1, Glutamate-Cysteine Ligase Modifier Subunit (GCLM), Solute Carrier Family 7 Member 11 (SLC7A11), and Sulfiredoxin 1 (SRXN1) proteins. Kamran *et al.* [76] investigated the functional properties of lupin protein hydrolysates from *L. angustifolius* after enzymatic digestion using

pepsin, pancreatin, and flavourzyme, which was followed by molecular weight cut-off separation. The discovery of ACE-inhibitory peptides in the 2–3 and 3–5 kDa fractions was achieved by monitoring biological activities. The ACE-inhibitory activities were highly significant for the most active fractions in this range, with IC₅₀ values ranging from 450 to 600 µg/mL. Gram-positive bacteria were considerably inhibited by lupin protein-derived peptide fractions, while Gram-negative bacteria were very slightly inhibited. The antibacterial properties of pancreatin hydrolyzed fractions were the highest, with several fractions showing 85% inhibition against *Bacillus cereus* and *Staphylococcus aureus*.

2.10 Integrated food formulation and incorporation of lupins into food products

Supplementing food products with lupin protein components can be a potent method to enhance nutritional value by boosting protein content while also offering the health advantages associated with intrinsic bioactive peptide sequences. Villacres *et al.* [77] investigated the breadmaking potential of lupin flour after debittering and solid-state fermentation. Dough rheology, technological and nutritional features of bread, as well as acceptability, were all examined at varying levels of lupin flour substitution (10, 15, and 20%). During the mixing process, lupin weakened the dough, resulting in a brief development time and decreased stability, particularly in fermented lupin flour. At higher substitution levels (> 10%), debittered lupin flour and fermented lupin flour had a substantial impact on the technological properties of lupin-wheat bread, affecting bread volume, crust luminosity, crumb cohesiveness, and robustness. When fermented lupin flour was used, the negative effects reported at the highest substitutions (20%) were reduced, albeit breads earned a lower score due to the acidic flavour detected by panelists. When compared to wheat bread, both lupin flours produced lupin-wheat composite breads with a similar composition but containing increased contents of proteins (0.8%), fat (2.4%), and dietary fibre (6.5%).

Similarly, the hydrolytic and glycemic indexes of the lupin-wheat composite bread were much reduced when compared to the 100% wheat bread.

Woldemariam *et al.* [78] investigated the potential production of ice cream from lupin and soymilk as alternatives to dairy product. The goal of this study was to see how varying quantities of blue lupin and soymilk substitution affected the physicochemical and sensory qualities of vanilla ice cream. The protein level of blue lupin (5.86%) was higher than that of soymilk (4.83%) and cow milk (3.4 %), according to raw material analysis. The protein content of blue lupin ice cream varied between 4.27 and 4.79%, whereas the highest protein content obtained for soymilk ice cream was 4.39%. The soymilk ice cream had a greater crude fat level than blue lupine ice cream, with 7.47 % and 6.6%, respectively. Sensory characteristics of the ice cream samples were assessed, and the taste and texture were found to be comparable to dairy ice cream at up to 25% replacement. However, when the amount of soy and sweet lupin in the ice cream increased, the panelists' preference scores declined. [78] Coban et al. [79] conducted an experiment to determine the influence of the cooking method on the glycemic index values of chips made from a lupin flour formulation. The glycemic index values of the chips with whole lupin flour added were found to be lower than those of the chips with de-hulled lupin flour added. There was a significant decrease in glycemic index values between the lupin flour-enhanced samples and the control samples (without lupin flour addition). The sample with the highest ratio of lupin flour had the lowest glycemic index value. In a similar study by Ozcan et al. [80] the sensory, colour parameters, acrylamide levels, fat absorption, peroxide number, and p-anisidine values of lupin chips made with various formulations (de-hulled lupin, whole lupin, wheat, and maize flours) were investigated. The addition of lupin flour significantly affected the p-anisidine value of the chips. The addition of lupin flour and the cooking method affected the final product's colour, fat absorption, and peroxide values. Chips

made with whole lupin flour were discovered to have distinct chemical properties from those made with de-hulled lupin flour. Acrylamide was not found in any of the formulations investigated.

Albuja-vaca *et al.* ^[81] also formulated a complementary food where rice flour (RF) was replaced with lupin flour (LF) to create gluten-free pasta. A total of seven response factors were examined. Mixture-process models ($p < 0.05$) described luminosity, ash content, protein content, chromium, color, weight gain, and loss of solids. Because of its high protein and mineral contents, LF was a significant factor. With 20 g/100 g LF, 30 g/100 g egg, and 0.15 g/100 g guar gum, the optimal formulation was achieved. Partially substituting RF for LF in gluten-free products was found to be a reliable alternative in this investigation. Leonard *et al.* ^[82] explored the potential of adding roasted LF to beef sausage. Before being ground into flour, *L. angustifolius* seeds were roasted, and then the flour soaked until it had the same moisture level as beef. Six distinct preparations of beef sausage were tested, with meat being replaced by hydrated LF ranging from 0% to 36% (w/w). The addition of LF improved the stability of the beef emulsion (fluid released fell from 9.35% to 1.53%) and reduced cooking loss from 22.70% to 14.30%. Incorporating lupin into this formulation resulted in a softer texture and improved adhesiveness. Sensory examination revealed no significant differences in overall appearance, aroma, flavor, or overall liking between the control and 12% lupin-enriched beef sausage formulations, implying that LF can be used at this level without compromising consumer acceptance. Moreover, the inclusion of LF in beef sausage could be cost-effective. ^[82]

2.11 Patent information

The database on patents for lupin is rapidly growing. Patents on lupin technologies particularly in the area of lupin protein extraction, functionality, health benefits/application, and food formulation make up the bulk of patents in the database. Patents are drivers for the innovative creation of

proteins with desirable techno-functional properties that are relevant to the industries. A brief overview of lupin patents filed in the database (United States of America, Canada, European Union, Japan, WIPO) is essential to showcase the work done so far and develop models relevant to the industries. Researchers are relentlessly working to unravel and create new patent technologies that will usher lupin into an area of pulse dominance.

Auger and Corre^[83] patent described a process for obtaining lupin flour with desirable solubility, stability, and emulsifying properties. The process involves grinding the cotyledon with a pin hammer and passing the product to a pair of smooth-pressing rollers to obtain flour that disperse readily in dry, pasty, or liquid medium. Humans are naturally averse to bitter tastes. Bitter substances in lupin seeds which are majorly alkaloids are hepatotoxic when consumed in large amounts. Hussmann's invention^[84] described a process for debittering lupin seeds. The process is energy efficient and retains the bulk of the nutritive composition of the seeds. The recovered bitter substance can also be channeled as a biologically active agent. The invention also describes a method for treating plants with the recovered bitter substance to assess how it affects plant growth. Wasche and colleagues' invention^[85] describes an efficient method for processing lupin seeds to obtain protein, oil, and alkaloids. The invention is characterized by the formation of a discoid flake, followed by the indirect application of heat coupled with extensive water exclusion. Lipid-reduced flakes and lipids are obtained via solvent extraction; finally, the lipid-reduced flakes are subjected to aqueous debitterization to produce an alkaloid-reduced raffinate. In contrast, Jaeggli's invention^[86] describes an extraction process carried out under atmospheric conditions to obtain proteins and anti-nutritive compounds. This invention can be channeled into the production of plant-based protein milk from lupin, protein hydrolysate, and ballast-enriched protein products.

Lupin bran can be channeled as a source of high-fiber food. Tucek's invention ^[87] pertains to a method for making food-grade lupin bran and a high-fiber food product that contains food-grade lupin bran. It relates specifically, but not solely, to the use of lupin bran in high-fiber breakfast and snack foods. The efficient extraction of lupin protein isolates is important in the use of the protein in foods and other relevant applications. Kaur and colleagues' invention ^[88] described a process for obtaining food-grade lupin protein extract, lupin fiber fraction, and lupin hydrocolloid fraction. Patents are also documented for the use of lupin as a food product base ^[89], food product precursor ^[90] and food product in combination with the chickpea-containing product ^[91]. The target food products are beverages, dairy, and soups. Johnson and Al-Ali's ^[92] invention modified lupin protein with ultrasound waves to improve its value as a protein feedstock. Compared to the unmodified lupin protein, the modified lupin protein had decreased thermal stability. This invention has valuable applications in the food processing industry. Innovative technology where lupin flour is processed into food products like a crunchy crisp with one or more essential amino acids is also on record. Van Den Einden's invention ^[93] describes a process for the formation of crunchy crisp with lupin flour in combination with oats and eggs. The sensory evaluation of the invention was promising. Duranti's invention ^[94] describes the formation of semolina-based pasta that has been enriched with lupin. The pasta advantageously keeps all the organoleptic and consistent qualities of traditional semolina pasta while having a protein content comparable to that of egg yolks or meat. The protein quality of complement food products from cereals and legumes is desirable when compared to cereals or legumes alone.

The search for a sustainable plant-based dairy substitute is important for individuals suffering from lactose intolerance. Snowden and colleagues' invention ^[95] describes the preparation of lupin protein-based dairy substitutes. These dairy substitutes made from an aqueous dispersion of lupin

proteins may be used to create derivative goods that are viable replacements for processed dairy milk products including ice cream, cheese, and yogurt. Bremer's invention ^[96] further detailed the production of lupin milk. Additionally, further processing of this product and the tofu-like foodstuff that results from them are discussed. Probiotics improve digestion, boost immunity, and in some cases aid in weight loss. Song et al invention ^[97] detailed a method for the preparation of probiotics fermented lupin products. The described invention has noticeable advantages over existing techniques, which include fermentation of the lupin seeds using a lactic acid bacteria-prepared direct-fed-type fermentation agent. The rich protein content in the lupin seeds is utilized, the majority of the dietary fibers and other nutritional ingredients are secured, and the lupin seeds are transformed into nutritional and healthy novel foods, all at a low cost and with minimal waste. The ability of lupin proteins to form colloidal suspension is well documented. Eisner et al. invention ^[98] detailed the formation of emulsions from lupin proteins with desirable rheological properties, taste, and color. This invention's emulsion can be used to make desserts and beverages including lupin pudding, yogurt, fresh cheese, and dairy substitute products. Fitchett et al. ^[99] developed highly functional lupin protein fractions with the ability to stabilize emulsions at a higher oil concentration compared to other vegetable proteins such as soybean. The emulsifying qualities are comparable to those of animal-derived proteins such as caseinates, which are more expensive and frequently problematic. This method is efficient for developing lupin protein emulsions with a high oil binding capacity. The invention of Fryirs et al ^[100] provides a composition of lupin proteins to be used as an emulsifier in the stabilization of oil-in-water emulsion for food products. The ideal protein content from this invention ranges from 70 to 98% (dry weight basis), and about 85 to 95% of the lupin protein's molecular weight is greater than 20 kDa.

Lupin protein extract has attracted attention as an agent that maintains or improves skin firmness and lessens or slows down the development of skin cellulite. Baudouin et al. ^[101] used lupin protein extract at a concentration of 0.001 to 30% dry weight to maintain or improve skin firmness. Application of lupin peptide extract topically to skin explants promoted the expression of collagens I and III, two main macromolecules of the dermal extracellular matrix, as well as collagen IV and laminin 5, markers of the epidermal-dermal interface. Therefore, lupin peptide extract can reinforce the epidermal-dermal junction and dermal extracellular matrix to increase the firmness and elasticity of the skin. Lupin peptide extract with anti-metalloprotease activities such as anti-collagenase and anti-gelatinase activity has important cosmetic, nutraceutical, and pharmaceutical applications. Msika et al. ^[102] utilized a hydrolyzed lupin peptide extract with anti-metalloprotease activity to combat inflammatory diseases such as ulcers, periodontosis, and arthrosis. The invention also detailed the use of the lupin peptide extract in cosmetic applications related to aging. Charagnac's invention ^[103] employed a combination of flavonoids, vegetable oil, and lupin peptides as a cosmetic for the treatment of edema and inducing skin regeneration. Researchers are exploring the potential of lupin protein extract on erythemas. Choulot et al patent ^[104] involves the application of lupin protein extract in conjugation with oligosaccharide as an inflammatory agent in the repair of cutaneous barriers, with the overall scope for the treatment of erythemas. Enzyme hydrolysis of lupin protein isolates yields hydrolysates which may possess bioactive properties. Millan et al patent ^[105] detailed a method to produce lupin protein hydrolysates by hydrolyzing lupin protein with alcalase. The hydrolysates obtained were used in the treatment of inflammatory diseases.

The search for a potent hypoglycemic agent will provide succor to individuals battling with diabetes. Morazzoni and Duranti's patent ^[106] describes the use of gamma conglutin as a

therapeutic agent in the treatment of type-2 diabetes. The patent detailed the overall efficient extraction process of γ -conglutin. γ -conglutin could be used in a pure form, as a concentrate, or as a mixture. Lupin γ -conglutin was investigated for its hypoglycemic effect in rats and compared to the antidiabetes drug metformin. Luzi and Teruzzi's invention ^[107] claims to induce muscle cell differentiation or muscle growth by extracting γ -conglutin from lupin seeds containing between 10 and 30% weight of the protein and exposing muscle cells or muscles to an effective amount of the extract. Other claims involving γ -conglutin involve the role it plays in insulin signaling, protein synthesis, and muscle cell metabolism ^[108]. The major allergenic protein found in lupin seeds is β -conglutin. Jimenez et al invention ^[109] provides an improved antibody technique and kit to detect and quantify β -conglutin, the primary allergen proteins of lupin seeds, in foodstuffs. The invention uses an ELISA technique and an antibody developed against an epitope shared by at least seven isoforms of the β -conglutin proteins of *L. angustifolius* and at least one of *L. albus*. Thus, the invention's approach is sensitive and specific enough to detect and quantify lupin residues in food samples that may cause allergic reactions in hypersensitive consumers. A similar innovation utilizes the lupin ITS1 gene as the identification target, and highly specific loop-mediated isothermal amplification primers are designed. ^[110] The lupin component in food allergens can be easily identified using the method described in the invention, which has good repeatability and sensitivity.

Although the foregoing patents collectively map the innovation landscape for lupin proteins, their significance is best understood not by what each claims, but by the specific technical hurdle each was devised to overcome. The earliest and most consequential barrier is the quinolizidine alkaloid content, which is bitter and hepatotoxic and historically confined lupin to animal feed; the value of the debittering and extraction inventions [84–86] therefore lies less in alkaloid removal per se

than in achieving it without the excessive water use, energy input, and soluble-nutrient loss that burden conventional aqueous debittering, with Wäsche's discoid-flaking and water-exclusion approach [85] and the recovery of alkaloids as a saleable co-product representing the genuine inventive steps. A second hurdle concerns functionality retention: because alkaline extraction and isoelectric precipitation partially denature the protein and lower solubility, the flour and isolation patents [83,88] advance mild mechanical processing and integrated co-fractionation of fiber and hydrocolloid streams to preserve dispersibility and valorize co-products, though the residual denaturation and the difficulty of scaling gentler salt-based extraction remain unresolved. The emulsifier patents [98–100] address the well-known underperformance of plant proteins relative to caseinate at high oil loadings, and their inventive contribution is the selective enrichment of high-molecular-weight fractions (70–98% protein, >20 kDa) capable of stabilizing high oil-phase emulsions, yet they leave pH sensitivity, allergenicity, and off-flavor largely unaddressed. The γ -conglutin therapeutic patents [106,107] confront a purification hurdle, since γ -conglutin does not precipitate at its isoelectric point and requires specialized isolation, while the outstanding barriers are economical scale-up and oral bioavailability of the intact protein. Finally, the allergen-detection inventions [109,110] overcome the challenge of sensitivity and specificity across multiple β -conglutin isoforms and both species through conserved-epitope antibodies and species-specific amplification primers, with matrix interference in processed foods remaining the chief limitation.

Table 2.5 Patents for lupin seed proteins

Patent	Brief description
Processing of lupin flour	Removal of bitter substances, alkaloids, antinutritive compounds, and oil. Production of high-grade lupin concentrates and isolates. [83-86]
Production of high-fiber food	Production of food-grade lupin product base rich in fiber and protein. Processing of lupin flour to products such as pasta, semolina, and crunchy chips. [87-94]
Dairy substitute	Production of lupin-based dairy substitutes and fermented lupin products that are a viable replacement for milk products including ice cream, cheese, and yogurt. [95-97]
Emulsions with lupin proteins	Detailed formation of emulsions with desirable rheological properties, taste, and color. [98-100]
Cosmetics	Utilization of lupin protein in cosmetic applications related to aging, increasing skin firmness and elasticity, slowing down the development of skin cellulite and erythemas. [101-104]
Hypoglycemic agent	Efficient extraction of gamma conglutin and investigation of the therapeutic activity in the treatment of type-2 diabetes. [106-108]
Bioactive peptides	Enzymatic hydrolysis of lupin protein isolates to obtain bioactive peptides with functional properties such as anti-inflammatory properties. [105]

2.12 CONCLUSIONS AND FUTURE RESEARCH

Lupin seeds have great potential in the food industry. The era where this plant is viewed as an underutilized legume is fast fading away. The numerous nutritional benefits such as high protein and fiber contents will in time push this plant to the limelight. The ideal extraction procedure to be employed when working with lupin depends on the target food product. Optimization of the extraction protocol is essential to be able to obtain a higher yield of protein. The techno-functional properties of lupin also make it suitable for incorporation into various food formulations. This is advantageous because aside from boosting the nutritional profile of the formulated foods, the proteins can also serve as a nutraceutical because of their intrinsic bioactive peptide sequences. The hypoglycemic activities of γ -conglutin fraction from lupin seeds are promising and it is an area that is ripe for exploration by the food and nutrition industry. Robust lupin protein patent technologies are also required to create protein fractions with functional properties that are relevant to the food, cosmetics, and pharmaceutical industries. It is hoped that further developments of lupin's wet and dry extraction technologies will lead to more protein yield and improved flavour to increase the economic value of lupin crop.

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CRedit AUTHORSHIP CONTRIBUTION

Stanley Chukwuejim: Writing-original draft, Methodology, Investigation, Formal analysis.

Alphonsus Utioh: Resources, Software, Validation, Writing-review & editing. Tristan D. Choi:

Resources, Funding acquisition, Writing-review & editing. Rotimi E. Aluko: Writing-review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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CHAPTER THREE
MANUSCRIPT TWO
PHYSICOCHEMICAL AND FUNCTIONAL PROPERTIES OF BLUE LUPIN AND
WHITE LUPIN PROTEIN ISOLATES

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3.0 ABSTRACT

White lupin (WLPI) and blue lupin (BLPI) protein isolates were compared for their composition and functional properties. WLPI showed higher protein yield (70.30%) than BLPI (66.47%), while BLPI had slightly higher crude protein content (86.96%). Both isolates had similar essential amino acid profiles, with sulfur-containing amino acids, valine, and lysine being limiting. WLPI flour exhibited larger particle sizes (D_{4,3} of 29.3 µm) and higher denaturation temperature (100 °C) than BLPI (D_{4,3} of 19.5 µm and 89 °C, respectively). However, BLPI demonstrated higher surface hydrophobicity (688.13 arbitrary units) than WLPI (656.77 arbitrary units). WLPI showed superior in vitro protein digestibility (84.21%) and oil-holding capacity (8.18 g/g). Analysis of sulfhydryl groups revealed higher free SH content in WLPI (27.22 µmol/g), while BLPI had higher total SH (86.11 µmol/g) and disulfide bond content (29.44 µmol/g). These differences in structural and physicochemical properties between WLPI and BLPI may significantly influence functional behavior in various food systems, potentially affecting their applications in food formulations.

Keywords

Lupin, protein isolate, polypeptide composition, thermal properties, physicochemical properties, functional properties

3.1 INTRODUCTION

The demand for alternative protein sources has intensified in recent years due to environmental concerns, health considerations, and the need to diversify protein options. Interest is growing in the use of underutilized legumes, such as lupins, as readily available protein sources. Lupins originated from the Mediterranean and Andean regions and have a rich history that dates back to ancient civilization. They were cultivated by ancient Egyptians, Greeks, and Romans primarily for the ability to improve soil fertility through nitrogen fixation¹ In recent decades, lupins have attracted renewed interest as a valuable source of high-quality protein for both human consumption and animal feed, particularly in the context of the growing global demand for plant-based proteins.² Lupin seeds contain 30-45% protein, comparable to soybeans, with a favorable amino acid composition that complements cereals in human and animal diets.³ Additionally, lupins are rich in dietary fiber, essential fatty acids, and various bioactive compounds, which contribute to their potential health benefits, including improved cardiovascular health, blood glucose regulation, and weight management.^{4,5} From an agricultural perspective, lupins are valued for their ability to thrive in marginal soils, drought tolerance, and role in crop rotation systems, by enhancing soil fertility and reducing the need for synthetic fertilizers.⁶

Fractionation and isolation of lupin proteins have been the subject of extensive research aimed at optimizing extraction methods to obtain protein isolates with enhanced functionality and purity. Common fractionation techniques include alkaline extraction followed by isoelectric precipitation, salt extraction, and ultrafiltration.⁷ The choice of the extraction method significantly influences composition and functional properties of the resulting protein isolates. For instance, alkaline

extraction tends to yield isolates with higher protein content but may alter the native structure of proteins, potentially affecting their functionality.⁸

pH and protein concentration significantly influence protein functional properties, playing crucial roles in food systems and processing.⁹ pH affects protein net charge, solubility, and conformational stability, thereby influencing properties like emulsification, foaming, and gelation.¹⁰ At pH values far from the isoelectric point, increased electrostatic repulsion enhances protein solubility and flexibility, often improving the emulsifying and foaming capabilities.¹¹ Protein concentration affects the availability of molecules for interfacial adsorption and network formation, thereby influencing emulsion stability, foam structure, and gel strength.¹² Higher concentrations generally improve these properties up to a critical point, beyond which saturation and overcrowding may occur. The interplay between pH and concentration is complex, and optimal conditions vary depending on the specific protein and the desired functionality.¹³

Recent studies have explored various physical modifications to enhance LPI's functional properties, including germination¹⁴, low-frequency ultrasound,¹⁵ and high-pressure homogenization.¹⁶ These treatments resulted in improved isolate production with enhanced functionality. Microbial fermentation of LPI has been shown to positively influence sensory and techno-functional properties, while reducing allergen potential and bitterness.¹⁷ Furthermore, the effects of heat treatment, pH, and NaCl on LPI's thermal and rheological properties have been investigated, revealing diverse functional attributes.^{18,19} LPI's role in gel structure development has been examined, particularly its interaction with fat particle surfaces, strengthening the structure of comminuted meat products.²⁰ Additionally, pH-dependent variations influence soluble lupin fractions' air-water interfacial properties, improving foaming capabilities and mechanical properties.²¹

The functional properties of lupin protein isolates, including solubility, water and oil holding capacity, foaming properties, and gelation, are crucial determinants of their applicability in food systems. These properties are influenced by various factors such as protein composition, extraction method, pH, ionic strength, and thermal treatment.²² The gelation properties of lupin proteins, primarily attributed to α -conglutin, are of interest for the development of meat alternatives and textured vegetable proteins. Heat-induced gelation of lupin proteins occurs at temperatures above 80°C, resulting in gels with varying hardness and water-holding capacity depending on protein concentration and processing conditions.²³ Our previous studies reported the potential of lupin protein isolates as alternative plant-based emulsifiers.²⁴ The work showed that the emulsifying properties of WLPI and BLPI were comparable to those of soybean protein isolates, with pH having a greater effect on emulsion formation than protein concentration. Despite the recognized potential of lupin proteins, there remains a significant knowledge gap regarding the specific structural and functional differences between protein isolates from different lupin species. The variability in functional properties among lupin species emphasizes the importance of species selection in developing protein ingredients with specific tailored characteristics. Existing research has largely focused on the individual functional characteristics of lupin species with limited comparative analyses.^{25–27} This study aimed to provide a detailed comparative analysis of blue and white lupin protein isolates by examining the differences and similarities in their physicochemical and foaming properties, which could contribute to further understanding of their functionality and potential use as ingredients in various food systems.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Blue (*Lupinus angustifolius* ‘Boregine’) and white (*Lupinus albus* ‘Dieta’) lupin seeds were donated by The Lupin Platform Inc. (Calgary, AB, Canada). All the reagents and chemicals used

for this study were of high-purity analytical grade and procured from either Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific Company (Oakville, ON, Canada). Reagents and chemicals were prepared using double-distilled water (DDW).

3.2.2 Methods

3.2.2.1 Production of lupin protein isolate

White and blue lupin protein isolates were extracted using a modified version of the protocol described by Osemwota et al.³² Lupin seeds were pulverized into a fine powder using a Retsch ZM200 ultra-centrifuge mill, operating at 14,000 rpm and equipped with a 0.5 mm ring sieve. The resulting flour underwent a defatting process where, for each 100 g of flour, 1000 mL of acetone was used. This mixture was agitated for 120 min, after which the liquid phase was removed. This step was performed twice, and the defatted flour left to dry in a ventilated fume cupboard for 12 hours. The defatted flour was dispersed in double-distilled water (DDW) at a 1:10 ratio (w/v). The suspension was adjusted to pH 9.0 using 1 M NaOH to facilitate protein solubilization. This mixture was subjected to continuous agitation for 60 min and centrifuged (5600 x g, 30 min). The resulting supernatant was filtered through a double layer of grade 90 cheesecloth (thread count: 40 × 36). The filtered solution was then adjusted to pH 4.5 using 1 M HCl. This acidified mixture was stirred for 60 min to allow for protein precipitation. The precipitated proteins were collected by centrifugation (5600 × g, 30 min). The precipitate underwent two washing cycles with DDW, each followed by centrifugation under the same conditions. The final protein isolate was adjusted to pH 7.0 and then lyophilized using VirTis Freeze Dryer (SP Scientific, Warminster, PA, USA) at -23° C for 72 h to obtain a dry powder. The gross yield was calculated using the formula;

$$\text{Gross yield (\%)} = \frac{\text{Weight of protein isolate (g)}}{\text{Weight of lupin flour (g)}} \times 100 \quad (1)$$

The protein yield (recovery) was calculated using the formula;

Protein Yield (%)

$$= \frac{\text{protein isolate weight (g)} \times \text{isolate protein content (\%)}}{\text{Weight of lupin flour (g)} \times \text{flour protein content (\%)}} \times 100 \quad (2)$$

3.2.2.2 Compositional analysis

The moisture, dry matter, crude protein, crude fiber, and ash contents of lupin protein isolates were determined using procedures recommended by the Association of Official Analytical Chemists. The American Oil Chemists' Society methodology was used to determine the crude fat content.²⁸

3.2.2.3 Amino acid composition

The amino acid composition of the lupin protein isolates was assessed using an HPLC system equipped with a pico-tag column, following digestion with 6 M HCl for 24 h.²⁹ The sulfur-containing amino acids (cysteine and methionine) and tryptophan were identified after performic acid oxidation and alkaline hydrolysis of the samples, respectively.³⁰

3.2.2.4 Gel electrophoresis

Blue and white lupin protein isolates were subjected to reducing and non-reducing polyacrylamide gel electrophoresis as previously described by Osemwota et al.³¹

3.2.2.5 Surface hydrophobicity

The surface hydrophobicity was measured according to a previous method.³² WLPI and BLPI were dissolved in 0.1 M sodium phosphate buffer (pH 7.0) and diluted to 0.005 to 0.025% (w/v) based on protein content. A 20 μ L aliquot of 1-anilino-8-naphthalenesulfonate (8 mM in 0.1 M sodium phosphate buffer pH 7) was added to 4 mL of each diluted isolate, and the fluorescence intensity (FI) at 390 nm excitation and 470 nm emission wavelengths was measured. The slope of the plot of FI versus protein concentration was calculated by linear regression analysis and used as an index of surface hydrophobicity.

3.2.2.6 Differential scanning calorimetry

Thermal properties of WLPI and BLPI were obtained using the TA Q100-DSC thermal analyzer (TA Instruments, Newcastle, DE). Each protein isolate, weighing 15 mg, was accurately placed in an aluminum pan, which was then sealed and heated from 40°C to 140°C at a rate of 10°C per minute. A sealed empty pan was used as a reference, and all experiments were repeated three times. The onset temperature (To), denaturation temperature (Td), and enthalpy of denaturation (ΔH) were calculated using the Universal Analysis 2000 software (Version 4.5).³¹

3.2.2.7 In vitro protein digestibility

In vitro digestibility of BLPI and WLPI was analyzed using previously described protocols,³³ with some modifications. Lupin protein samples (6.25 mg protein/mL) were each suspended in an aqueous solution containing deionized water and adjusted to pH 8 with 0.1 M NaOH while stirring at 37 °C. A 3 mL aliquot of an enzyme solution containing 1.6 mg trypsin, 3.1 mg chymotrypsin, and 1.3 mg peptidase per mL was added to 30 mL of each protein suspension. The reductions in pH of the mixture were recorded every 30 s over a 10 min period using a pH meter. The percentage protein digestibility of each protein sample was calculated using the regression equation proposed by Hsu et al.³²:

$$\% \text{ Protein digestibility (Y)} = 210.46 - 18.10X_f \quad (3)$$

Where X_f is the final pH value of each lupin sample after digestion for 10 min.

3.2.2.8 Intrinsic fluorescence

Intrinsic fluorescence measurements were performed using an FP-8350 spectrofluorometer (Jasco Co., Japan). To prepare the stock solutions, 10 mg protein/mL of each lupin protein sample were dispersed in 0.1 M acetate (pH 3 and 5), phosphate (pH 7), or Tris-HCl (pH 9) buffers. Following centrifugation, the supernatant was collected and diluted to 0.002% (w/v) using the appropriate buffers. The protein samples were excited at 280 nm (tyrosine and tryptophan) and emissions were

recorded from 300 to 500 nm. To obtain sample fluorescence spectra, the emissions of the buffer blanks were subtracted from those of the respective samples. $F_{\max}$ is the maximum fluorescence intensity (FI) obtained during the wavelength scan and $\lambda_{\max}$ is the wavelength at $F_{\max}$.

3.2.2.9 Particle size distribution

The particle size distribution (PSD) of BLPI and WLPI flour dispersions was determined using laser diffraction (Mastersizer 3000, Malvern Instruments Ltd., Worcestershire, UK). Lupin protein isolates were dispersed in ultrapure water in 50 mL centrifuge tubes at a concentration of 1% protein (w/v) and adjusted to pH 7 by adding 1 M NaOH solution. The samples were then shaken overnight at 4 °C. The particle refractive index was set to 1.45, the absorption index was 0.1, and the dispersant (water) refractive index was 1.33. Protein dispersions equilibrated to room temperature, were introduced into the dispersing unit using ultrapure water as the dispersant until a laser obscuration of 8 - 12% was achieved. The results are presented as volume-weighted mean particle diameters ($D_{4,3}$), surface area weighted mean particle diameters ($D_{3,2}$), and volume percentiles ($D_v(10)$, $D_v(50)$, and $D_v(90)$).

3.2.2.10 Zeta potential

The surface charge (zeta potential) was determined according to the method described by Asen and Aluko.³⁴ The zeta potential of lupin protein isolates was measured at pH 7 using a Zetasizer nano-Z instrument (Malvern Instruments Ltd., UK). The lupin samples (0.1% w/v based on protein content) were dispersed in 0.1 M phosphate buffer pH 7. The samples were shaken overnight at 4 °C and then equilibrated to room temperature. Subsequently, insoluble materials were removed by centrifugation ($2000 \times g$ for 10 min at room temperature). The measurement was performed using automatic voltage selection, and the zeta potential calculated using the Smoluchowski model, with a refractive index of 1.45 and absorption of 0.001.

3.2.2.11 Sulfhydryl group (SH) and disulfide linkages (SS)

The SH and SS were determined according to a previous method.³⁴ Briefly, Tris-Gly buffer (pH 8.0) containing 0.004 M EDTA and 8 M urea was used to prepare protein dispersions (7.5 mg/mL each) and were stirred overnight at room temperature. To determine the free SH content, 1 mL of the stirred protein dispersion was added to 4 mL Tris-Gly buffer and 0.05 mL Ellman's reagent. The mixture was incubated for 20 min at 25 °C and the absorbance measured at 412 nm. To determine the total SH content (free SH and reduced SS), 1 mL of the stirred protein dispersion was mixed with 4 mL Tris-Gly buffer and 0.05 mL 2-mercaptoethanol (ME). The mixture was incubated for 1 h at 25 °C and then subjected to three stages of centrifugation and resuspension to remove the ME. The final precipitate was suspended in 10 mL of Tris-Gly buffer and 0.04 mL Ellman's reagent was added to 4 mL of the suspension. The absorbance was measured at 412 nm after 20 min of incubation.

3.2.2.12 Least gelation concentration (LGC)

The method of Malomo and Aluko³⁵ was used to determine the LGC of BLPI and WLPI. A series of aqueous protein solutions was prepared in 0.1 M acetate buffer (pH 3), 0.1 M phosphate buffer (pH 5 and 7), or 0.1 M Tris buffer (pH 9) at concentrations ranging from 2% to 20% (w/v, based on protein weight). The mixtures were placed in test tubes, vortexed for 5 min, and heated in a water bath at 95 °C for 1 h. The tubes were immediately cooled to room temperature and stored at 4 °C for 14 h. LGC was determined as the lowest protein concentration at which the gel did not slip when the tube was inverted.

3.2.2.13 Water and oil holding capacity

The water and oil-holding capacities of BLPI and WLPI were determined using a previously described method³⁵ with some modifications. To determine the oil-holding capacity (OHC), 40 mg/mL protein isolates were measured in pre-weighed 15 mL centrifuge tubes and 5 mL of canola

oil added. The sample dispersion was vortexed for 1 min and allowed to stand for 30 min at room temperature. The mixture was centrifuged at 5600 x g for 30 min, and the supernatant containing excess oil was drained for 15 min. The centrifuge tubes were then reweighed to determine the amount of oil retained per gram of protein. For water holding capacity, the canola oil was replaced with buffers at different pH values (3, 5, 7, and 9) and the whole process repeated as described above.

3.2.2.14 Foam properties

Foam properties of BLPI and WLPI were determined according to the method described by Osemwota et al.³¹ with some adjustments. Varied lupin protein concentrations (10, 15, and 20 mg protein/mL) were dispersed in 0.1 M acetate (pH 3 and 5), phosphate (pH 7), or Tris-HCl (pH 9) buffers and the resulting mixtures vortexed for 1 min and left to hydrate for 30 min. The foam was generated in a 50 mL graduated centrifuge tube using a 20 mm shaft on a Polytron PT 3100 homogenizer (Kinematica AG, Lucerne, Switzerland) at 20,000 rpm for 1 min, and its volume measured to determine the foaming capacity.

Foam Capacity (FC)

$$= \frac{(\text{volume after homogenization} - \text{volume before homogenization})}{(\text{volume before homogenization})} \times 100 \quad (4)$$

The volume of foam remaining after 30 min at room temperature was then expressed as a percentage of the original foam volume to evaluate foam stability.

3.2.2.15 Statistical analysis

Triplicate measurements were used to obtain values that are reported as mean $\pm$ standard deviation. IBM SPSS statistical software (version 28) was used to perform the analysis of variance, and the values were tested for statistical significance ($p < 0.05$) using Duncan's multiple range test.

3.3 RESULTS AND DISCUSSION

3.3.1 Compositional analysis

The proximate composition results are shown in Table 3.1. The crude protein contents of WLPI and BLPI are comparable to the previously reported results for *L. luteus*, *L. angustifolius* cv Jurien, and *L. albus* cv Murringo.^{27,36,37} There was no significant difference ($P < 0.05$) in the crude fat content for both lupin protein isolates, and the low-fat content resulted from the defatting process's efficiency before the protein isolation. Furthermore, the low crude fiber content (≤ 0.07 g/100 g) of both lupin protein isolates indicates that they were effectively removed during the protein isolation process. The lower moisture content of WLPI is more desirable than that of BLPI in the formulation of products with improved shelf life during prolonged storage. The ash content, which is the mineral-rich residue obtained after sample burning, is significantly ($P < 0.05$) higher in WLPI (7.47 g/100 g) compared to BLPI (3.23 g/100 g). High mineral content often influences protein functional properties such as water-holding capacity and gelation.³⁸ Peng et al.³⁹ demonstrated that divalent ions, such as calcium, can shield electrostatic interactions between charged protein molecules. Additionally, these ions facilitate cross-linking by binding to negatively charged carboxylic acid groups on adjacent protein molecules, forming salt bridges that modify protein functionality.

3.3.2 Amino acid composition

The amino acid composition data of WLPI and BLPI (Table 3.2) provided information on the nutritional properties and quality of the lupin protein isolates. The nutritional value of a protein source is largely determined by its amino acid profile, which directly affects the ability to meet dietary requirements. These requirements are defined as the quantity and quality of amino acids necessary to support metabolic processes efficiently, maintain optimal body composition, and promote growth.⁴⁰ WLPI has more sulfur-containing amino acids, methionine, and cysteine, than BLPI. The two sulfur-containing amino acids are regarded as endogenous antioxidants because they protect against oxidative damage.⁴¹ In eukaryotic organisms, methionine is the initiating

Table 3.1 Yield and proximate composition of white (WLPI), and blue (BLPI) lupin protein isolates*

Parameters	WLPI (g/100 g)	BLPI (g/100 g)
Protein yield	70.30 ± 0.98 ^a	66.47 ± 2.20 ^b
Gross yield	26.89 ± 0.37 ^a	24.66 ± 0.81 ^b
<i>Proximate composition</i>		
Crude protein (N x 5.7)	85.67 ± 0.02 ^b	86.96 ± 0.01 ^a
Moisture	4.93 ± 0.05 ^b	6.57 ± 0.11 ^a
Crude fiber	0.07 ± 0.07 ^a	0.06 ± 0.04 ^a
Crude fat	0.09 ± 0.07 ^a	0.03 ± 0.01 ^a
Total ash	7.47 ± 0.58 ^a	3.23 ± 0.08 ^b
Non-fibre carbohydrate	1.77 ± 0.30 ^b	3.15 ± 0.00 ^a

*Values represent mean ± standard deviation. Values with different superscripts within the same row are significantly different (p<0.05) as determined by ANOVA and Duncan's multiple range test. Non-fibre carbohydrate was calculated by difference (100-Σmoisture, protein, crude fiber, crude fat, total ash).

amino acid in the biosynthesis of proteins, while the ability of cysteine to form disulfide bonds plays an important role in protein structure and folding.⁴² BLPI, with a methionine and cysteine amino acid score of 64%, is deficient in the two amino acids. This suggests that BLPI may be less effective as a stand-alone protein source in meeting the sulfur-containing amino acid requirements of humans and may need to be complemented with other protein sources rich in methionine and cysteine to achieve a balanced amino acid profile. Branched-chain amino acids (BCAAs) valine, leucine, and isoleucine play crucial roles in protein metabolism and various physiological processes. BCAAs regulate protein synthesis and degradation by acting as a carbon source of oxidation for energy production and as a precursor for muscle protein synthesis.⁴³ In different health conditions such as cancer, liver diseases, sepsis, and surgery leading to muscle wasting, BCAA supplementation reduced muscle protein breakdown and improved protein synthesis.⁴⁴ Both isolates exhibited similar valine amino acid scores, with WLPI at 76% and BLPI at 74%, indicating moderate deficiencies in this essential amino acid. This suggests that additional valine supplementation with complementary proteins may be beneficial when incorporating these isolates into food formulations to meet dietary requirements. The isoleucine and leucine amino acid scores for the WLPI and BLPI are higher than the reference FAO/WHO requirements for adults. As expected, the most abundant amino acids in both lupin isolates are glutamic acid, aspartic acid, and arginine. Aspartic acid and glutamic acid are catabolized during different metabolic processes to provide a steady supply of amino groups, whereas arginine plays a particularly significant role because it is involved in the biosynthesis of several metabolic active compounds including creatine, nitric oxide, glutamate, polyamines, proline, and glutamine.^{45,46} Beyond its role as a building block for proteins, arginine is recognized as a functional amino acid with diverse physiological benefits, such as reducing oxidative stress, enhancing antioxidant capacity, boosting

immune function, and supporting the development of bone and muscle tissues.⁴⁷ The amino acid profiles in this study are similar to other literature reports for lupin species and cultivars.^{36,48}

Protein yield, which represents the percentage of total protein recovered in the isolate relative to the protein content in the starting material, and gross yield, which indicates the total mass of protein isolate recovered relative to the initial flour mass, were evaluated for both lupin varieties.

White lupin protein isolate (WLPI) demonstrated superior protein yield ($70.30 \pm 0.98\%$) compared to blue lupin protein isolate (BLPI) ($66.47 \pm 2.20\%$). This trend was also reflected in the gross yield, where WLPI showed significantly higher values ($26.89 \pm 0.37\%$) than BLPI ($24.66 \pm 0.81\%$). The higher protein recovery in WLPI suggests that proteins from white lupin may be more readily extractable under the alkaline solubilization and isoelectric precipitation method. The results are similar to the protein yield (75.5%) and gross yield (27.1) reported by Jayasena et al.,⁴⁹ for isoelectric precipitated lupin protein

Table 3.2 Amino acid profile and score of white (WLPI) and blue (BLPI) lupin protein isolates*

Indispensable/conditionally indispensable amino acid	WLPI		BLPI	
	Level (g/100g Protein)	AA Score %	Level (g/100g Protein)	AA Score %
Histidine	2.04 ± 0.12 ^b	128 ^α	2.36 ± 0.00 ^a	148 ^β
Threonine	2.59 ± 0.01 ^a	103	2.41 ± 0.02 ^a	96 ^α
Lysine	3.66 ± 0.01 ^a	76 ^α	3.6 ± 0.02 ^a	75 ^α
Tyrosine	4.07 ± 0.04 ^a		2.88 ± 0.09 ^b	
Methionine + Cysteine	2.03 ± 0.02 ^a	88 ^β	1.48 ± 0.04 ^a	64 ^α
Valine	3.04 ± 0.03 ^a	76 ^α	2.95 ± 0.02 ^a	74 ^α
Isoleucine	3.77 ± 0.03 ^a	126 ^β	3.52 ± 0.04 ^a	117 ^α
Leucine	6.79 ± 0.07 ^a	111 ^β	5.95 ± 0.03 ^a	98 ^α
Phenylalanine	3.34 ± 0.03 ^a		3.32 ± 0.04 ^a	
Phenylalanine + Tyrosine	7.41 ± 0.04 ^a	181 ^β	6.20 ± 0.13 ^b	151 ^α
Tryptophan	0.53 ± 0.02 ^a	80 ^α	0.77 ± 0.01 ^a	117 ^β
Cysteine	1.44 ± 0.00 ^a		0.96 ± 0.02 ^a	
Dispensable amino acids				
Serine	4.18 ± 0.04 ^a		3.7 ± 0.01 ^a	
Arginine	8.43 ± 0.04 ^b		9.13 ± 0.13 ^a	
Glycine	2.53 ± 0.02 ^a		2.7 ± 0.03 ^a	
Aspartic acid	8.87 ± 0.00 ^a		8.29 ± 0.03 ^a	
Glutamic acid	19.88 ± 0.04 ^a		19.39 ± 0.01 ^a	
Proline	3.17 ± 0.02 ^a		3.17 ± 0.03 ^a	
Alanine	2.19 ± 0.00 ^a		2.26 ± 0.00 ^a	

*Values are represented as mean ± standard deviation. Values with different superscripts (letters and symbols) within the same row are significantly different (p<0.05) as determined by ANOVA and Duncan's multiple range test.

3.3.3 Gel electrophoresis

Figure 3.1 presents the SDS-PAGE analysis of WLPI and BLPI under non-reducing and reducing conditions. Under non-reducing conditions (lanes B and C), both WLPI and BLPI displayed a complex pattern of protein bands, indicating a diverse range of protein subunits. This complexity is consistent with the known composition of lupin proteins, which primarily consists of storage proteins, mainly globulins. The major fractions of lupin seed proteins are α -, β -, δ -, and γ -conglutins, with α - and β -conglutins comprising approximately 80% of the total protein content. The bands observed in the 45-52 kDa and 20-22 kDa ranges likely correspond to the acidic and basic subunits of α -conglutin, respectively. These subunits are typically linked by disulfide bridges in native proteins. The presence of bands in the 17-64 kDa range is consistent with the expected molecular weights of β -conglutin monomers, which include low-, intermediate-, and high-molecular-weight subunits. The presence of these proteins is consistent with previous research reports on legume protein composition, confirming the structural similarities between lupin proteins and those from other legumes.^{3,31,50,51} Under reducing conditions (lanes D and E), both isolates showed changes in their band patterns. For BLPI, more intense bands appeared at approximately 22 kDa and 40 kDa, suggesting the dissociation of disulfide bonds and the separation of α -conglutin into its subunits. This effect was more pronounced in WLPI, indicating a potentially higher content of disulfide-linked proteins or a structure that is more susceptible to reduction. Notably, the SDS-PAGE profiles revealed distinct differences between the WLPI and BLPI. The WLPI lanes (B and E) showed a more complex and diffuse pattern, with a greater number of bands across a broader molecular weight range. This may be attributed to the heterogeneity of the protein subunits of white lupin, which could result from genetic diversity or differences in post-translational modifications. In contrast, the BLPI lanes (C and D) presented a more defined banding pattern, suggesting a more homogeneous protein composition. The

differences observed between the protein profiles of WLPI and BLPI may explain some of their distinct functional properties, such as gelation and foaming.

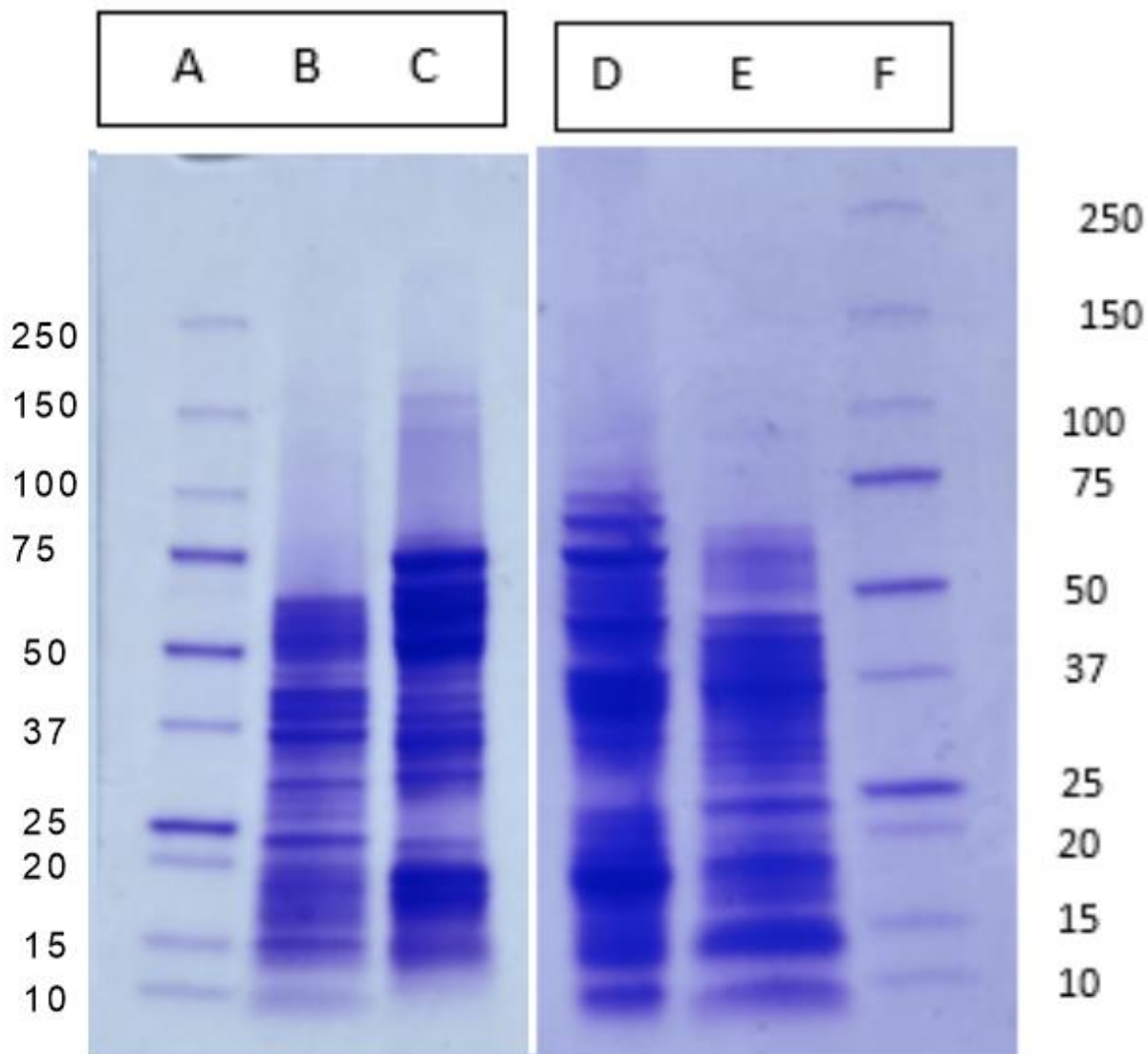


Figure 3.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of white (WLPI) and blue (BLPI) lupin protein isolates under non-reducing and reducing conditions. **A:** Standard protein markers (kDa); **B:** non-reduced WPLI; **C:** non-reduced BLPI; **D:** reduced BLPI; **E:** reduced WLPI; **F:** Standard protein markers (kDa).

3.3.4 Surface hydrophobicity

Surface hydrophobicity is an important indicator of a protein's ability to interact with non-polar substances, such as oils and fats, and plays a key role in determining the emulsifying, foaming, and gelation properties of proteins.⁵² In this study, the surface hydrophobicity values for WLPI and BLPI were determined to be 656.77 and 688.13, arbitrary units, respectively (Table 3.3). These results revealed a statistically significant difference between the two isolates' surface hydrophobicity, with BLPI exhibiting higher surface hydrophobicity than WLPI. The higher surface hydrophobicity observed in BLPI suggests that it may have a greater capacity to form and stabilize emulsions and foams than WLPI. The difference in surface hydrophobicity between WLPI and BLPI can be attributed to several factors. These may include variations in the amino acid composition, particularly the ratio of hydrophobic to hydrophilic amino acids, as well as differences in protein conformation and degree of protein unfolding during the isolation process.⁵³ The higher surface hydrophobicity of BLPI might indicate greater exposure of hydrophobic residues on the protein surface, which suggests greater effect on its protein conformation (compared to the WLPI) during the alkaline extraction and isoelectric precipitation method used to generate the protein isolates. The results agree with those of Vogelsang-O'Dwyer et al.³, who found that BLPI has higher surface hydrophobicity than WLPI, which was attributed to the prevalence of smaller particles in BLPI.

Table 3.3 Surface hydrophobicity, thermal, functional, and sulfhydryl group and disulfide linkages of white (WLPI) and blue (BLPI) lupin protein isolates*

Parameters	WLPI	BLPI
<i>Surface hydrophobicity (S_o)</i>		
S_o (arbitrary units)	656.77 ± 5.00^a	688.13 ± 10.28^b
<i>Thermal properties</i>		
T_o Onset ($^{\circ}\text{C}$)	96.04 ± 0.41^b	84.13 ± 1.75^a
Maximum T_d ($^{\circ}\text{C}$)	100.81 ± 0.76^b	89.31 ± 1.03^a
ΔH Area (J/g)	2.18 ± 0.32^a	0.64 ± 0.45^b
<i>Functional properties</i>		
IVPD (%)	84.21 ± 0.27^a	82.76 ± 0.27^b
OHC (g/g)	8.18 ± 0.41^a	7.57 ± 0.34^b
<i>Sulfhydryl group (SH) and disulfide linkages (SS)</i>		
Free SH ($\mu\text{mol/g}$)	27.22 ± 0.26^a	25.40 ± 0.05^b
Total SH ($\mu\text{mol/g}$)	93.07 ± 1.88^a	86.11 ± 2.32^b
Disulfide bond ($\mu\text{mol/g}$)	33.83 ± 0.92^a	29.44 ± 1.23^b

Values are represented as mean $\pm$ standard deviation. Values with different superscripts within the same row are significantly different ($P < 0.05$) as determined by ANOVA and Duncan's multiple range test. T_o : onset denaturation temperature; T_d : maximum denaturation temperature; ΔH : enthalpy of denaturation; S_o : surface hydrophobicity; OHC: oil holding capacity; IVPD: *In-vitro* protein digestibility.

3.3.5 Differential scanning calorimetry

The thermal properties of protein isolates, such as onset temperature (T_o), maximum denaturation temperature (T_d), and enthalpy change (ΔH), are critical for understanding their stability and functionality in various applications. These properties are crucial for understanding how lupin protein isolates can be used in various thermal applications. As shown in Table 3.3, the WLPI had a significantly higher onset temperature (96.04°C) than BLPI (84.13°C). This indicates that WLPI begins to denature at higher temperatures, suggesting greater thermal stability. The higher onset temperature of WLPI implies that it may retain its native structure better during moderate heat treatments, which could be advantageous in certain food processing applications where

maintaining the protein structure is important. The maximum denaturation temperature for WLPI (100.81 °C) was also significantly higher than BLPI (89.31 °C). The higher T_d of WLPI suggests that it can withstand more intense heat treatments before complete denaturation occurs, potentially better preserving its functional properties under high-temperature processing conditions. The differences between WLPI and BLPI highlight the variability in thermal properties among lupin species and emphasize the importance of species selection in developing protein ingredients with specific thermal characteristics. The higher thermal stability of WLPI may be attributed to differences in protein composition, possibly due to a higher proportion of heat-stable proteins or stronger intramolecular interactions.⁵⁴ WLPI also has a higher content of disulfide bonds (Table 3.3), which may have contributed to the higher thermal stability in comparison to the BLPI. When compared to other studies on lupin protein isolates, the results for WLPI and BLPI are consistent with previously reported thermal properties, where onset temperatures ranged from 80°C to 95°C and maximum denaturation temperatures from 85°C to 100°C, depending on lupin variety and processing conditions.^{25,26,36} Similar studies on other legume protein isolates, such as lentils, peas, and chickpeas, also provide a comparative framework.^{55,56} Soy protein isolates typically show onset temperatures of approximately 70°C to 90°C and maximum denaturation temperatures between 80°C and 100°C, positioning the WLPI's thermal stability at a comparable level.⁵⁷

3.3.6 In vitro protein digestibility (IVPD)

As shown in Table 3.3, the IVPD results for WLPI (84%) and BLPI (82%) provide valuable insights into these plant-based proteins' nutritional quality and potential bioavailability. These values indicate that both lupin protein isolates have relatively high digestibility, which is a positive attribute for their use in food and human nutritional applications. Factors contributing to the high digestibility of these lupin protein isolates may include the effective removal of anti-nutritional

factors during the isolation process, and potential partial denaturation during processing, which can increase enzyme accessibility and the inherent structural characteristics of lupin proteins that favor enzymatic hydrolysis. Lqari et al.⁷ reported IVPD values for blue lupin protein isolates ranging from 86% to 94%, which are higher than the values observed in the current study. The higher digestibility in the previous work might be due to differences in protein isolation methods (extraction with sodium sulfate) or differences in the lupin varieties used. Boye et al.⁵⁸ reported the digestibility of various legume protein isolates such as pea (66-82%), chickpea (63-89%), and lentil (73-95%). The IVPD values for WLPI and BLPI in our study are comparable to those of other legume protein isolates, positioning lupin proteins favorably on the plant protein market.

3.3.7 Intrinsic fluorescence

The fluorescence spectra of BLPI and WLPI at different pH values are shown in Figure 3.2. Fluorescence spectroscopy is a sensitive technique that measures the emission of light from excited fluorophores, primarily tryptophan and tyrosine residues in proteins.⁵⁹ The intensity and wavelength of fluorescence emission are influenced by the local environment of these aromatic amino acids, making them an excellent tool for studying protein structure and conformational changes.⁶⁰ At pH 3, both BLPI and WLPI exhibited high fluorescence intensity with peaks at approximately 320-330 nm, characteristic of tryptophan fluorescence. The higher intensity of WLPI suggests that its tryptophan residues may be less exposed to environmental quenching agents than BLPI.⁶¹ The absence of a distinct tyrosine emission peak (typically around 308 nm) indicates that tyrosine and tryptophan residues are likely in close proximity, allowing efficient energy transfer to tryptophan.⁵⁹ Significant reductions in fluorescence intensity were observed at pH 5 for both isolates. This could indicate protein aggregation or conformational changes that exposed the aromatic amino acids to a more hydrophilic environment, leading to fluorescence

quenching.⁶² The absence of peaks suggests a major structural rearrangement near the isoelectric point of these proteins. At pH 7, the fluorescence intensity increased again, with BLPI showing a higher peak than WLPI. This difference suggests that BLPI and WLPI have distinct conformational arrangements at neutral pH, potentially influencing their functional properties differently in food systems. The highest fluorescence intensities were observed at pH 9 for both isolates, with BLPI slightly higher than WLPI. The peaks were slightly red shifted compared to lower pH values, which could indicate increased exposure of tryptophan residues to a more polar environment.⁶³ The increased intensity at pH 9 suggests structural conformations that moved the tryptophan residues closer to each other.

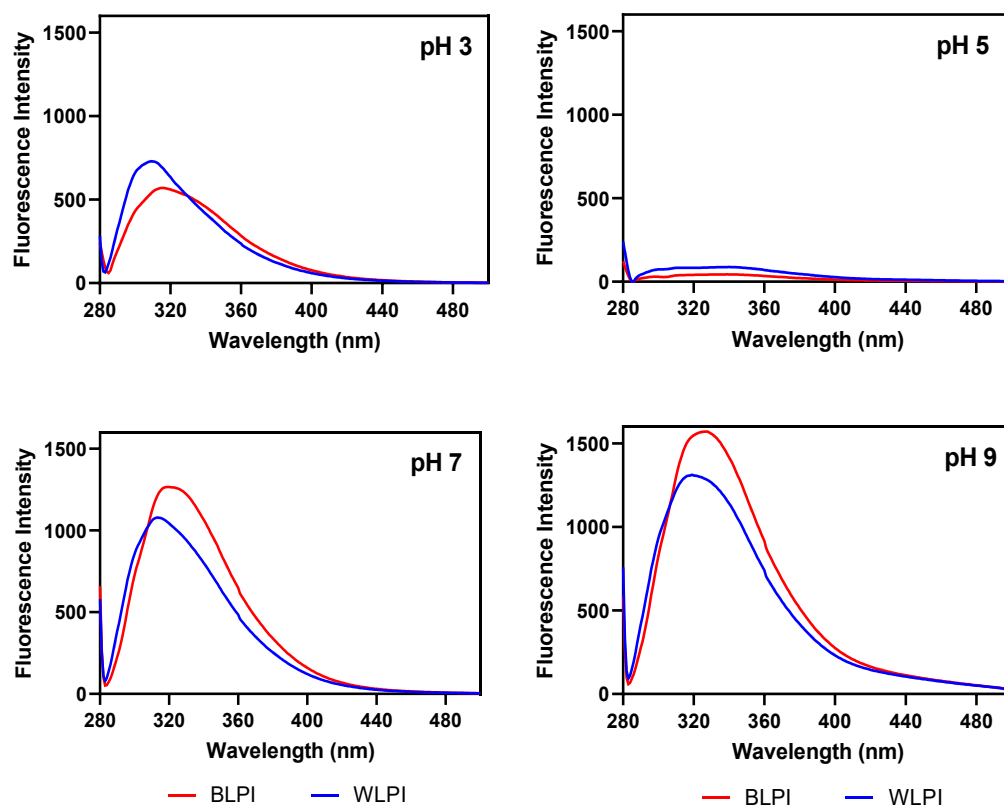


Figure 3.2 Intrinsic fluorescence intensity of white and blue lupin protein isolates. Samples (2 μ g protein per mL) were excited at 280 nm and emission was measured from 290 to 500 nm. WLPI-White lupin protein isolate; BLPI-Blue lupin protein isolate.

3.3.8 Particle size distribution

The particle size distributions of WLPI and BLPI protein isolates revealed significant differences in their physical characteristics, which can influence their functional properties and applications in food systems. The volume-weighted mean diameter $D[4,3]$ for WLPI (29.30 μm) is notably larger than that of BLPI (19.50 μm), indicating that WLPI particles are generally larger (Table 3.4). These differences suggest that WLPI may have undergone more protein-protein interactions during isolation, which led to the formation of bigger aggregates. The distribution percentiles showed that 90% of WLPI particles ($D_x(90)$) are below 60.70 μm , while for BLPI, this value is considerably lower at 37.90 μm . Interestingly, the $D_x(10)$ values are relatively close (6.43 for WLPI and 5.83 μm for BLPI), suggesting similarities in the smallest particle fractions. In addition, the WLPI displayed a broader distribution with a peak shifted towards larger particle sizes, whereas BLPI exhibited a narrower distribution with a peak at smaller particle sizes (Figure 3.3). These differences in particle size distribution can significantly affect functional properties such as solubility, dispersibility, gelation, and water- and oil-holding capacities.³ The large particle size of WLPI may enhance the formation of a stronger gel network due to increased protein-protein interactions^{64,65}. In contrast, BLPI's smaller particles may contribute to better foaming properties by balancing the surface area and mobility at the air-liquid interface. Additionally, WLPI's large particles, despite a lower surface area to volume ratio, may possess porous cavities and create void spaces that enhance oil entrapment, resulting in higher oil holding capacity. The narrower distribution of BLPI suggests more uniform particles, which could lead to a more consistent performance in food applications. In contrast, the broader distribution of WLPI may offer a range of functionalities within a single product. Thus, the choice between WLPI and BLPI should be guided by the specific application and desired textural outcomes in the final product, emphasizing the importance of particle size distribution in food formulations.

Table 3.4 Particle size parameters for white and blue lupin protein isolates

Particle size distribution (μm)	WLPI	BLPI
D [4,3]	29.3 ± 0.31 ^a	19.50 ± 0.25 ^b
D [3,2]	14.60 ± 0.19 ^a	11.50 ± 0.04 ^b
Dx (90)	60.70 ± 0.54 ^a	37.90 ± 0.82 ^b
Dx (50)	23.90 ± 0.36 ^a	15.20 ± 0.06 ^b
Dx (10)	6.43 ± 0.08 ^a	5.83 ± 0.02 ^a

Values are represented as mean ± standard deviation. Values with different superscripts within the same row differ significantly ($P < 0.05$) as determined by ANOVA and Duncan's multiple range test.

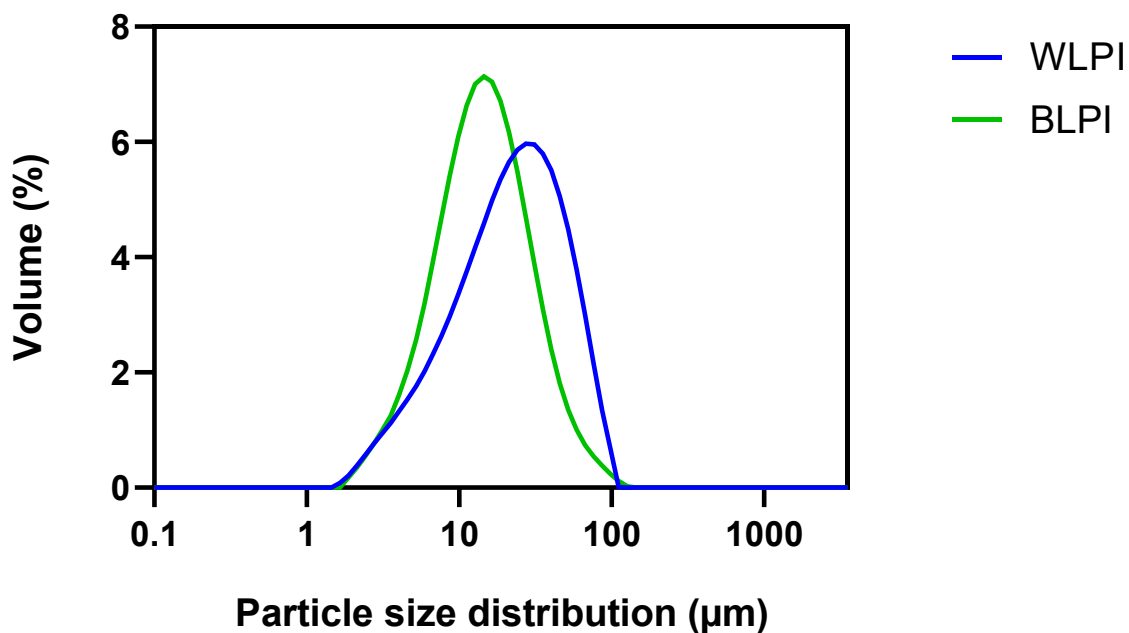


Figure 3.3 Particle size distribution of white and blue lupin protein isolates. WLPI- White lupin protein isolate; BLPI- Blue lupin protein isolate.

3.3.9 Sulfhydryl group (SH) and disulfide linkages (SS)

When subjected to heat treatment, proteins undergo structural and conformational changes. This process begins with denaturation, where the native structure of the protein unfolds, exposing previously hidden reactive groups and sulfur-containing amino acids. As the protein molecules unfold, they become more prone to interactions with other proteins, particularly through hydrophobic regions previously concealed within the protein core. The exposed thiol groups of cysteine residues can react with each other to form new disulfide linkages between the protein molecules.⁶⁶ The results shown in Table 3.3 reveal that WLPI had a slightly higher content of free SH groups than BLPI. This suggests that WLPI may have more reactive thiol groups available for interactions. The presence of exposed sulfhydryl (SH) groups in a protein indicates significant conformational alterations. This may be a result of the unfolding of the protein structure, which reveals previously hidden SH groups, or the breaking of existing disulfide (SS) bonds during processing. Higher free SH content can contribute to better solubility, gelation properties, and increased reactivity in food systems.⁶⁷ WLPI showed a higher content of disulfide bonds than BLPI. The higher SS content in WLPI indicates more intramolecular and intermolecular crosslinking within its protein structure. Higher disulfide bond content generally contributes to increased protein stability, as the result has a direct relationship with gelation and heat stability.

3.3.10 Zeta potential

The zeta potential is a measure of the surface charge of particles in a colloidal system and is indicative of the stability of such systems. Higher absolute values of zeta potential (either positive or negative) typically signify better stability, as particles with higher charges repel each other more strongly, thereby reducing the likelihood of aggregation. The zeta potential results at pH 7 as presented in Figure 3.4 for WLPI and BLPI indicate moderate negative surface charges. BLPI showed a slightly more negative value, suggesting a higher net surface charge. These values are

less negative compared to other legume proteins reported in the literature, which typically range from -15 to -30 mV for pea, faba, and chickpea isolates⁶⁸ and -20 to -40 mV for soy isolates.⁶⁹ The implications of these moderate zeta potential values may include limited colloidal stability, as values between -30 mV and +30 mV may lead to aggregation, the potential for weaker protein-protein repulsion, affecting functional properties, and influence protein-ingredient interactions in complex food systems. These moderate negative charges can influence the behavior of the isolates in food applications, potentially affecting their stability, solubility, emulsification, and foaming properties. At pH 3, both WLPI and BLPI exhibit positive charges, with WLPI having a slightly lower value than BLPI, indicating protonation of surface groups at acidic pH. At pH 5, which is close to the isoelectric point of lupin proteins, the zeta potential approaches zero, signifying minimal net surface charge, which promotes aggregation and reduced stability. At pH 9, the zeta potential becomes highly negative for both WLPI and BLPI, indicating deprotonation of amino acid functional groups and increased electrostatic repulsion, which enhances colloidal stability. These findings are similar to the study carried out by Chang et al.,⁷⁰ where proteins from green peas and chickpeas demonstrated a shift from positive to negative zeta potential with increasing pH due to changes in charge distribution. Such behavior impacts protein functionality, particularly solubility, and foaming, where high zeta potential at pH 9 enhances dispersion and foam stability, while low potential near the isoelectric point reduces functionality.

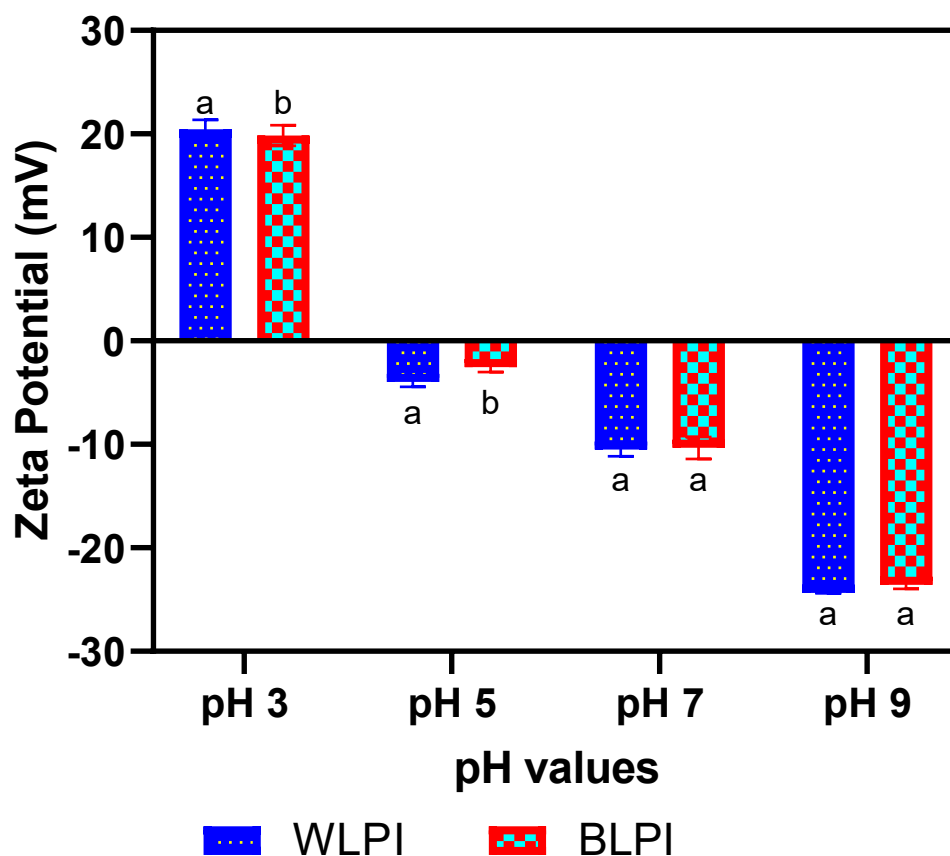


Figure 3.4 Effect of pH on the Zeta potential of white and blue lupin protein isolates. At each pH, bars with different letters have significantly different ($p < 0.05$) mean values as determined by ANOVA and Duncan's multiple range test.

3.3.11 Least gelation concentration

The least gelation concentration (LGC) is the minimum concentration of a protein solution required to form a gel under specific conditions. Thermal gelation of globular proteins occurs in four main steps: heat-induced unfolding of the tertiary structure, exposure of internal groups, aggregation of unfolded proteins to minimize energy, and formation of a continuous network.⁷¹ This process transforms the protein solution into an elastic gel structure, reflecting the reorganization of the proteins in response to thermal energy. The LGC values of WLPI and BLPI

at different pH values are shown in Figure 3.5. Both WLPI and BLPI have varying gelation properties across different pH levels, highlighting the importance of pH in protein gelation. At pH 5, WLPI and BLPI had similar LGC values, whereas at pH 3, WLPI had a slightly higher LGC than BLPI. At pH 7 and 9, BLPI shows significantly higher LGC values compared to WLPI. This may be due to the higher net surface charge as shown by the higher zeta potential of the BLPI (Table 3.3), which would reduce the intensity of protein-protein interactions needed to make a firm protein gel. LGC remained relatively consistent across all pH values for WLPI, with a slight increase at pH 9. At the same time, for BLPI, there was a marked increase in LGC at pH 7 and 9, indicating poor gelation properties under alkaline conditions. The LGC trends are related to the pH-dependent changes in the fluorescence spectra. For instance, the higher fluorescence intensity of BLPI at pH 7 and 9 aligns with its higher LGC at these pH values, possibly because of a more unfolded structure with reduced capacity to form defined protein networks.

The difference in gelation behavior between WLPI and BLPI, especially at neutral and alkaline pH, may be a result of several factors, such as variations in protein structure and amino acid composition, differences in heat stability of both lupin isolates, and inability to form new disulfide bonds. Compared with previous studies, lupin protein gels are weaker and softer than pea and soybean protein gels.^{23,72} Berghout et al.²³ pointed out that when compared to soybean protein isolate, lupin protein isolate's compact and thermally resistant structure inhibits the formation of intermolecular disulfide bonds while promoting intramolecular crosslinking. Although small, sulfur-rich proteins are present in the surrounding solution, their low concentration prevents them from effectively reinforcing the particle network.

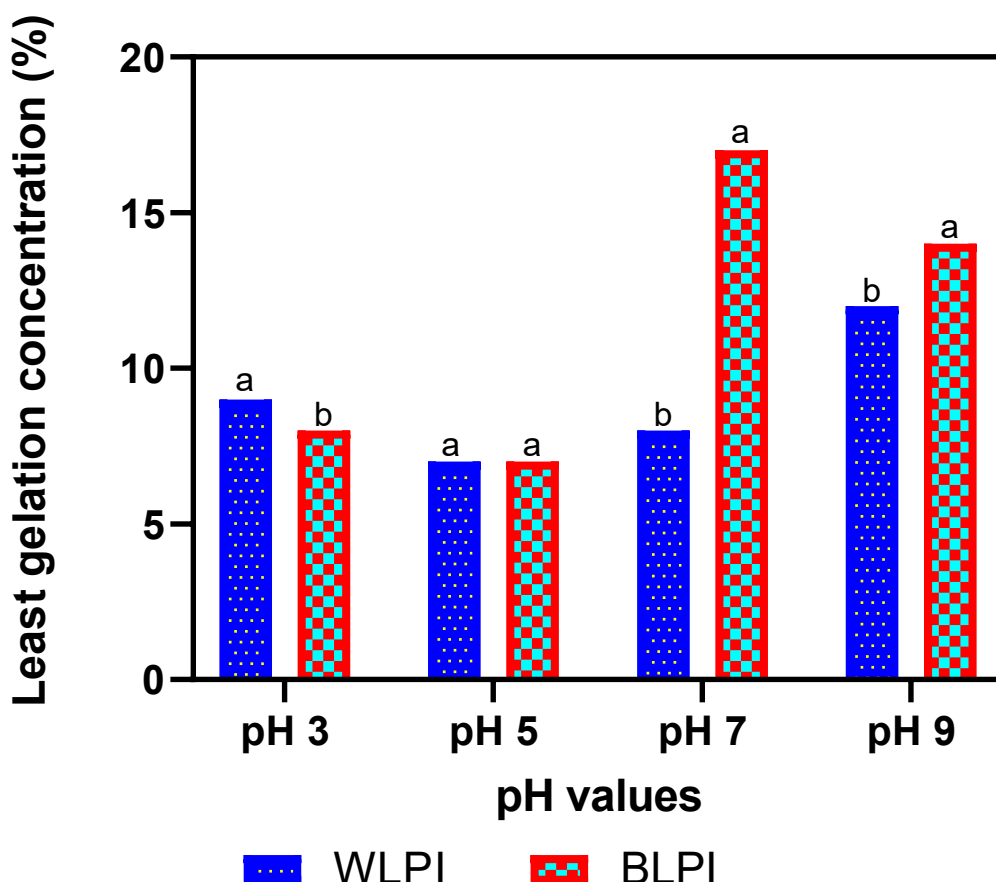


Figure 3.5 Effect of pH on the least gelation concentration of white and blue lupin protein isolates. At each pH, bars with different letters have significantly different ($p < 0.05$) mean values as determined by ANOVA and Duncan's multiple range test.

3.3.12 Water and oil holding capacity

The water holding capacity (WHC) of WLPI and BLPI, at different pH values, is shown in Figure 3.6. The WHC of both lupin protein isolates varied significantly with pH. The highest WHC was observed at pH 3 and 5, with a slight decrease at pH 7, and the lowest at pH 9. WLPI showed a higher WHC than BLPI at pH 3, whereas at pH 5 and 7, the WHC values were similar for both isolates. At pH 9, BLPI showed a slightly higher WHC than that of WLPI. The higher WHC at acidic and neutral pH values suggests that these protein isolates might be more suitable for food applications in these pH ranges, as they can retain more water, potentially improving the texture,

mouthfeel, and shelf life of food products. The pH-dependent changes in WHC suggest that protein structure and charge distribution are affected by pH, influencing their ability to interact with water molecules.²² WLPI had a slightly higher OHC (8.18 g/g) than BLPI (7.57 g/g), which could be due to the difference in the amino acid composition and greater surface hydrophobicity of WLPI in addition to the bigger particle size. The higher WHC and OHC of these protein isolates, especially at acidic and neutral pH, make them potentially suitable for use in various food products where water and oil retention are important, such as emulsions, meat alternatives, and baking products.

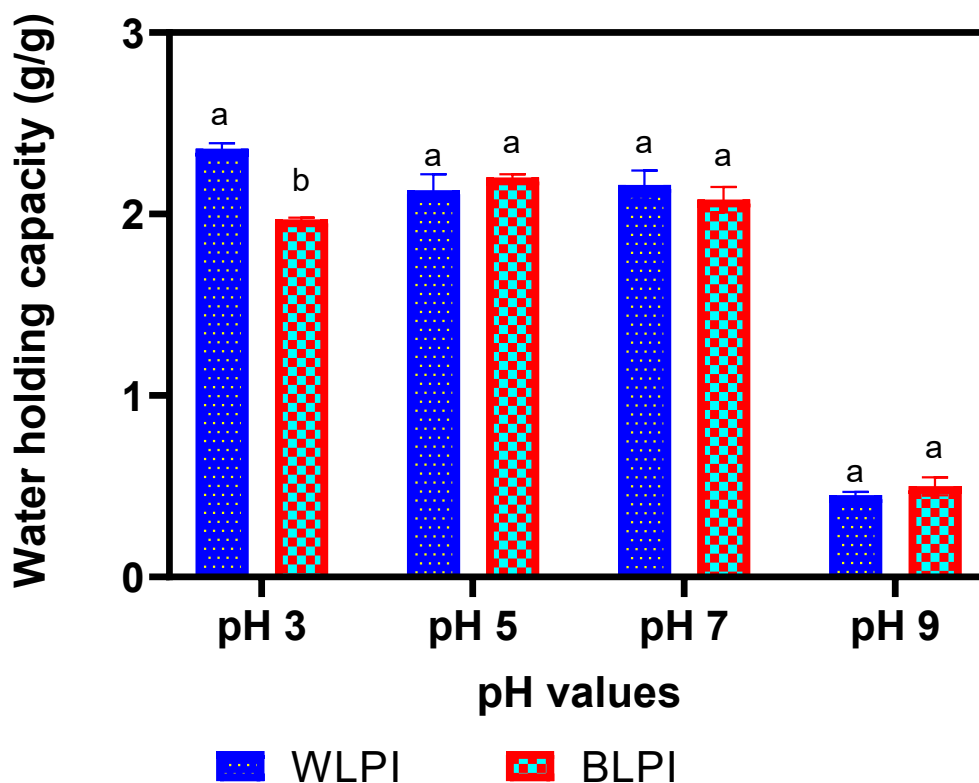


Figure 3.6 Effect of pH on the water-holding capacity of white and blue lupin protein isolates. At each pH, bars with different letters have significantly different ($p < 0.05$) mean values as determined by ANOVA and Duncan's multiple range test.

3.3.13 Foam capacity and stability

The foaming properties of WLPI and BLPI as evaluated across different pH values and protein concentrations are presented in Figure 3.7. The foam capacity results showed that both WLPI and BLPI exhibit pH-dependent foaming behavior. At pH 3, BLPI shows superior foam capacity compared to WLPI across all concentrations tested. This trend is consistent at pH 7 and 9, where BLPI maintains higher foam capacity. However, at pH 5, both isolates display similar foam capacities, with WLPI slightly outperforming BLPI at higher concentrations. The enhanced foam capacity of BLPI, particularly at pH 3, 7, and 9, suggests that it may have a greater ability to rapidly assemble at the air-water interface and form a strong viscoelastic membrane via protein-protein interactions.⁷³ Interestingly, both isolates show improved foam capacity as pH increases from acidic to alkaline conditions. This trend is particularly pronounced for BLPI, which exhibits its highest foam capacity at pH 9. This difference in foaming properties between BLPI and WLPI can be attributed to their solubility patterns as we previously reported.²⁴ For BLPI at pH 9, where the proteins are most soluble, proteins migrate faster to the air-water interface, effectively lowering the interfacial tension, thereby facilitating rapid foam formation. The high foaming properties exhibited at pH 5, which is close to the isoelectric point where the proteins are least soluble,²⁴ maybe a result of the insoluble protein acting as a Pickering stabilizer, thereby forming a rigid barrier around the foam bubbles. This pH-dependent behavior aligns with observations from other studies on legume proteins, where alkaline conditions often favor foam formation.⁷⁴ Protein concentration is an important factor influencing the foaming properties of lupin protein isolates, with higher concentrations enhancing these properties. At pH 3, there is a slight improvement in foaming capacity at 20 mg/mL, particularly for BLPI. However, the low solubility of lupin proteins at this pH may impede the formation of stable foams. At pH 5, there is no significant change in foaming capacity across the three different protein concentrations for BLPI. In contrast, at pH 9,

both WLPI and BLPI exhibit their highest foaming capacities. The influence of protein concentration is especially noticeable, with 20 mg/mL producing the best results. The improved solubility of lupin proteins at this alkaline pH likely enhances their ability to form stable foams. Overall, the improved foaming properties at 20 mg/mL can be attributed to the increased availability of protein molecules, which help stabilize the air-water interface and effectively reduce surface tension, forming a protective protein film around air bubbles.^{21,75}

Foam stability results (Figure 3.8) reveal that both WLPI and BLPI generally produce stable foams across the pH range tested, with most samples maintaining stability above 60% after the monitoring period. This suggests that both isolates can form strong interfacial films that effectively encapsulate air bubbles.⁷⁴ BLPI demonstrates superior foam stability at pH 7, particularly at higher concentrations, which could be advantageous for applications at neutral pH. WLPI, on the other hand, shows better foam stability at pH 5, especially at higher concentrations. This difference in the optimal pH for foam stability between the two isolates could be related to variations in their isoelectric points or structural characteristics, such as the ratio of hydrophobic amino acids and particle size. At pH 3 and 9, both isolates exhibited comparable foam stability, with slight variations depending on concentration. Generally, the high foam stability across the pH range indicates that both WLPI and BLPI can form cohesive viscous membranes required to maintain stable foams through reduced foam drainage. For both isolates, increasing the protein concentration generally led to an improved foam capacity and stability. This trend is consistent with findings from other studies on legume proteins, where higher protein concentrations typically resulted in enhanced foaming properties.⁷⁶ The pH-dependent behavior and generally high foam stability of both isolates suggest that they could be suitable for various food applications where foaming is desired, such as non-dairy desserts and certain bakery products.

In conclusion, this comparative study of WLPI and BLPI protein isolates revealed significant differences in structural and functional properties, providing useful information for their potential applications in food systems. The higher protein yield and thermal stability of WLPI, coupled with its superior IVPD and oil-holding capacity, suggest that it may be more suitable for heat-processed food applications. Conversely, BLPI's higher surface hydrophobicity, zeta potential, and foaming properties, potentially make it suitable for use in cold-processed foods or beverages. The larger particle size distributions of WLPI favor the formation of more robust gel structures, whereas BLPI offers better dispersion and smoother textures in liquid formulations. Both lupin protein isolates displayed a desirable high protein content; however, they were limited to sulfur-containing amino acids, valine, and lysine. These findings are important, as they offer food manufacturers valuable information for selecting the most appropriate lupin species for specific product applications, potentially leading to improved functional and nutritional properties in plant-based foods. However, the limitations of this study include the focus on only two lupin species and the absence of in vitro dynamic simulated digestibility studies. Additionally, the impact of long-term storage on the functionality of protein isolates has not been explored extensively. The potential consequences of these findings could lead to more targeted use of lupin proteins in the food industry, contributing to the diversification of plant protein sources and addressing the growing demand for sustainable non-allergenic protein alternatives. Future research should focus on the interaction of protein isolates with other macromolecules and food additives, investigating their performance in actual food systems, and exploring their potential health benefits.

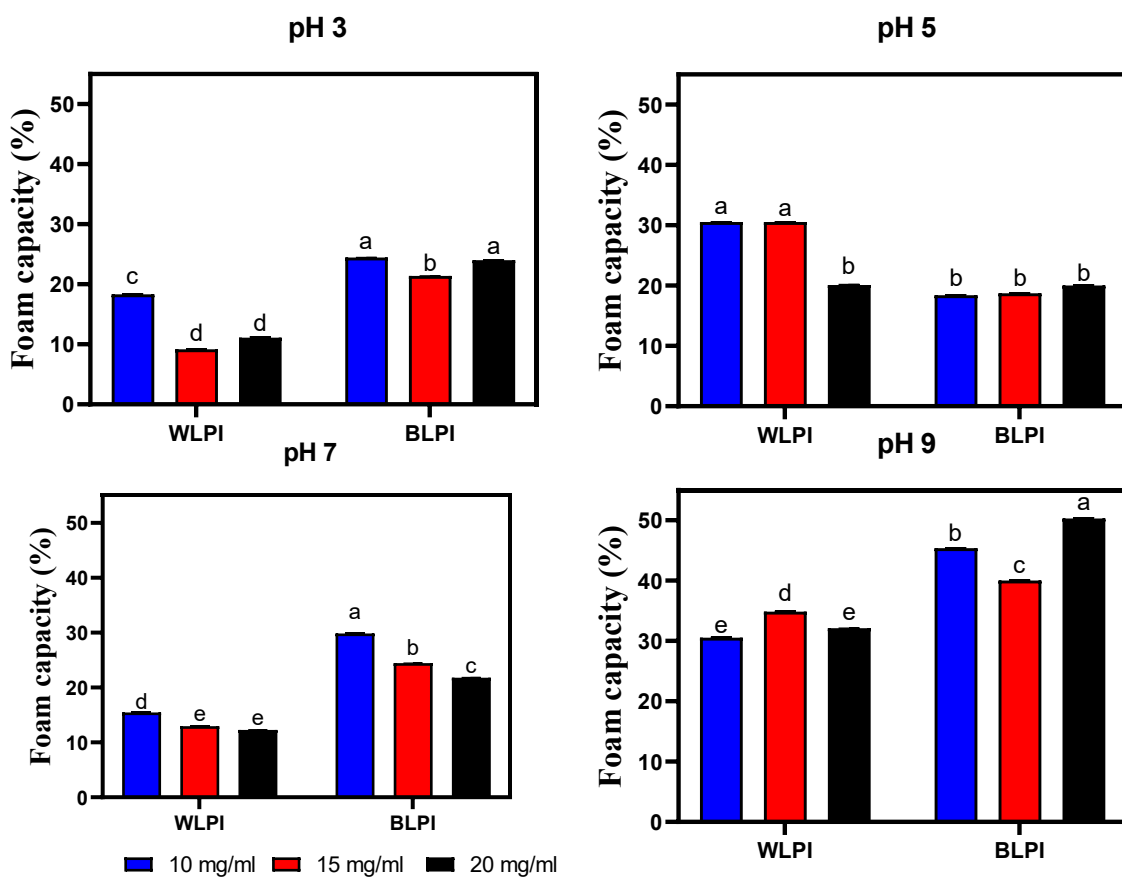


Figure 3.7 Effect of pH and protein concentration on the foam capacity of white and blue lupin protein isolates. At each pH, bars with different letters have significantly different ($p < 0.05$) mean values as determined by ANOVA and Duncan's multiple range test.

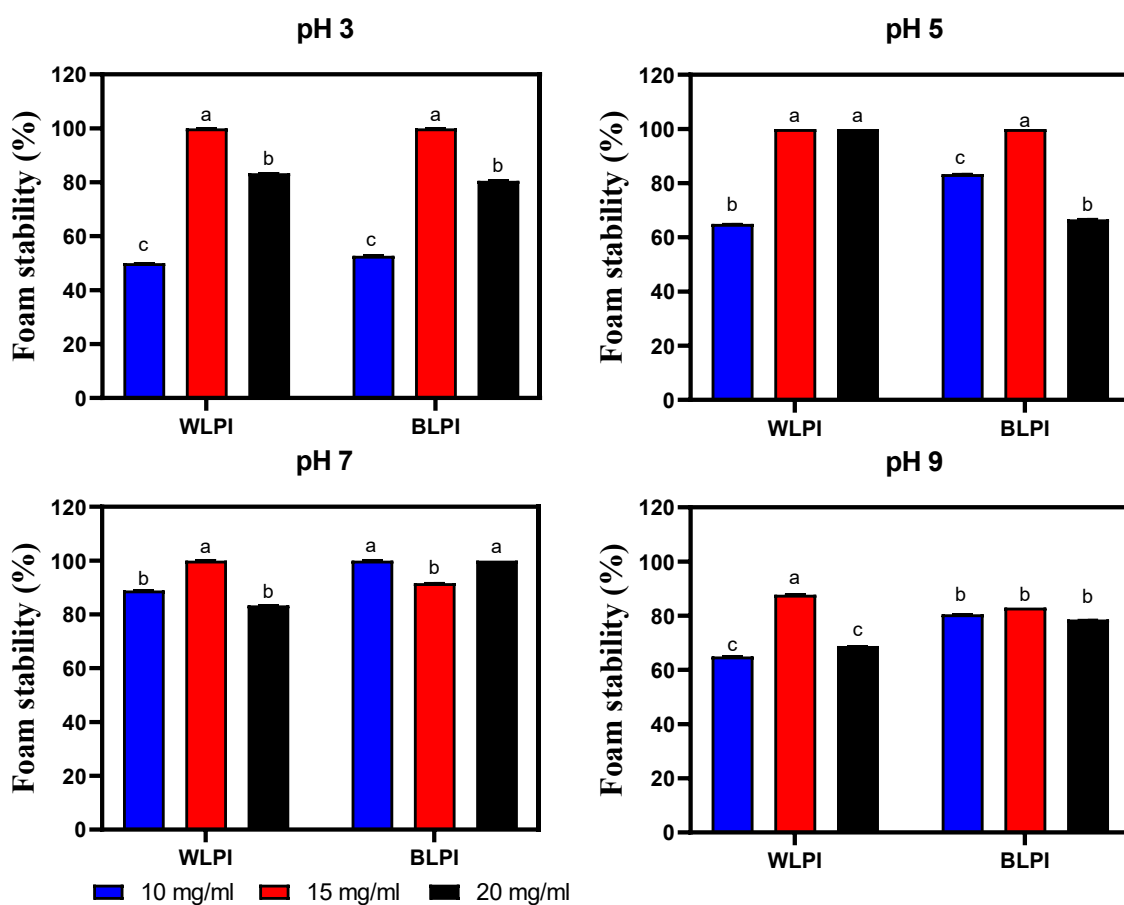


Figure 3.8 Effect of pH and protein concentration on the foam stability of white and blue lupin protein isolates. At each pH, bars with different letters have significantly different ($p < 0.05$) mean values as determined by ANOVA and Duncan's multiple range test.

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AUTHORSHIP

R.E.A Conceived, designed, supervised, reviewed, and edited the manuscript's final draft, S.C. carried out the research, analyzed the data, and wrote the first draft of the manuscript.

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TRANSITION STATEMENT

This chapter provided a comprehensive characterization of the physicochemical and functional properties of white lupin (*L. albus*) and blue lupin (*L. angustifolius*) protein isolates produced by alkaline solubilization and isoelectric precipitation. The results revealed significant species-dependent differences in amino acid composition, surface hydrophobicity, thermal stability, particle size distribution, zeta potential, sulfhydryl group content, gelation behavior, water- and oil-holding capacity, and foaming properties. Notably, BLPI exhibited higher surface hydrophobicity and smaller particle sizes, whereas WLPI showed greater thermal stability and a lower minimum gelation concentration. Both isolates demonstrated high protein content but were limited in sulfur-containing amino acids, valine, and lysine. These findings established a foundational understanding of the structural and functional distinctions between the two lupin species at the whole-isolate level. However, while Chapter 3 evaluated a broad range of techno-functional properties, the emulsifying behavior of the two lupin protein isolates was not examined in detail. Given that emulsification is one of the most commercially relevant functional properties for plant protein ingredients, a focused investigation is warranted. Furthermore, meaningful interpretation of lupin protein emulsifying performance requires comparison with an established industry-standard emulsifier. Therefore, Chapter 4 (Manuscript 3) builds directly on the findings of Chapter 3 by conducting a detailed comparative study of the emulsifying properties of BLPI, WLPI, and soybean protein isolate (SPI) across a range of pH conditions and protein concentrations, with particular emphasis on oil droplet size, emulsion microstructure, flocculation, and coalescence behavior.

CHAPTER FOUR
MANUSCRIPT THREE
**COMPARATIVE STUDY OF THE EMULSIFYING PROPERTIES OF BLUE LUPIN,
WHITE LUPIN, AND SOYBEAN PROTEIN ISOLATES**

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4.0 ABSTRACT

This study investigated the emulsifying properties of isoelectric pH-precipitated blue lupin (BLPI), white lupin (WLPI), and soybean (SPI) protein isolates at various protein concentrations and pH values (3, 5, 7, and 9). The solubility, secondary, and tertiary structure of the protein isolates at different pH were determined. The emulsion characteristics evaluated included the droplet size, microstructure characterization, coalescence index (CI), and flocculation index (FI). The solubility of the protein isolates was influenced by pH, with the lowest solubility recorded at pH 4–5. At pH 5, all the protein isolates had more β -sheet structure than α -helix, while at pH 9, the isolates contained the helical conformation more than β -sheet. In general, mean oil droplet size ($d_{3,2}$) was bigger at pH 3 and 5 ($\sim 5 - 16 \mu\text{m}$) when compared to pH 7 and 9 ($< 6 \mu\text{m}$). The presence of small spherical and uniform oil droplet emulsions ($< 9 \mu\text{m}$) stabilized by WLPI, BLPI, and SPI at pH 7 and 9 was confirmed using confocal laser scanning microscopy. Oil droplet coalescence was not the most prominent mechanism of emulsion instability for both lupin isolates and SPI, but rather bridging flocculation, with the FI highest at pH 5 (50-100%).

Keywords

Lupinus albus, *Lupinus angustifolius*, emulsions, coalescence, flocculation

4.1 INTRODUCTION

The search for sustainable plant-based emulsifiers has gained significant momentum in recent years, driven by the increasing consumer demand for healthier and environmentally friendly food options. Therefore, lupin seeds derived from *Lupinus* spp. have emerged as a promising source of proteins with unique functional and nutritional properties (Chukwuejim, Utioh, Choi, & Aluko, 2023). Among the diverse lupin species, blue (*Lupinus angustifolius*) and white (*Lupinus albus*) lupins have received significant attention because of their distinctive protein composition and functional properties, which offer several advantages as emulsifiers, including their plant-based origin, balanced amino acid profiles, and potential hypoallergenicity (Cabrita et al., 2023; Vogelsang-O'Dwyer et al., 2020). Lupin proteins have various applications in human food, including sausage production, bread, pasta, and extruded foods, and are primarily related to enhancing texture and color (Adem, Sadik, Worku, & Neela, 2019; Karoui, Terras, Yeddes, Hammami & Abderrabba, 2023; Villacrés, Cueva, Diaz & Rosell, 2020). Additionally, their emulsifying properties make them suitable for creating egg-free food emulsions like mayonnaise or salad dressing (Cabrita et al., 2023). However, limited research exists on the functional properties of lupin proteins when compared to soybean proteins, which are commonly used as vegetable-based emulsifiers. Soy protein isolate (SPI), mainly composed of β -conglycinin (7S) and glycinin (11S), is a highly functional food ingredient with high nutritional value that has been widely used in the food industry because of its excellent water solubility, emulsification, film formation, and gelation properties (Xu, Yu, Xue & Xue, 2023; Zhao et al., 2023).

Interactions between proteins and other biomolecules can be influenced by their structure-dependent functionalities. This is exemplified by the tendency of hydrophobic groups in proteins to interact with lipids and lipid-soluble compounds (Van de Vondel, Janssen, Wouters, & Delcour,

2023). Modifications to the protein environment, such as alterations in pH, temperature, ionic strength, and other factors, have been observed to frequently induce structural modifications in proteins. These alterations can lead to changes in a protein's biological, physicochemical, and functional properties (Nwachukwu & Aluko, 2018). Emulsification is widely recognized as a crucial aspect of the functionality of food proteins. During emulsification, proteins play a crucial role in stabilizing scattered oil droplets by reducing the interfacial tension between the oil and water phases (Francisco, Santos & Cunha, 2023, Mekala, Silva, & Saldana, 2022). This is achieved by producing a protein layer around the droplets, effectively preventing coalescence. Additionally, proteins contribute to the formation of a robust viscoelastic membrane, further enhancing emulsion stability by preventing oil droplet coalescence (Grasberger, Hammershoj & Corredig, 2023).

Although various studies in recent years have examined the structural, functional, and emulsifying properties of proteins from inexpensive and underutilized oil seeds and legumes, including canola (Vahedifar & Wu, 2022), pea (Adebiyi & Aluko, 2011), faba (Krause et al., 2023), and flaxseed (Nwachukwu & Aluko, 2018), there is a dearth of literature on the effects of pH changes on the structure and emulsifying properties of lupin protein. Thus, given the structural changes that food proteins undergo during processing, the current study aimed to understand the impact of varying pH conditions on changes in the conformation and emulsifying properties of lupin proteins. Although white lupin and blue lupin are both from the same *lupinus* genus, there are differences in their nutritional composition, amino acid profile, and mineral elements found in both species (Vogelsang-O'Dwyer et al., 2020). White lupin tends to have higher protein yield and oil content than blue lupin. Although both lupin proteins have been explored for their potential in food applications owing to their high protein content and nutritional value, detailed insights into their emulsification mechanisms, such as interfacial tension reduction, microstructure characterization,

droplet size stabilization, and the role of specific protein fractions, are limited. Characterization of these properties could shed light on the underlying mechanisms and potential applications of blue and white lupin protein isolates as emulsifiers in food systems. By elucidating the emulsifying capabilities of blue and white lupin proteins when compared to soybean proteins, we aim to contribute to the growing body of knowledge surrounding plant-based emulsifiers and their practical utilization in the food industry. The findings of this research will not only deepen our understanding of the unique functional properties of lupin proteins but also provide valuable insights into their potential application as ingredients in various food formulations.

4.2 MATERIALS AND METHODS

4.2.1 Materials

White (*Lupinus albus* 'Dieta') and blue (*Lupinus angustifolius* 'Boregine') lupin seeds were donated by The Lupin Platform Inc. (Calgary, AB, Canada). Soybean seeds were purchased from a local grocery store. All the reagents and chemicals used for this study were of high-purity analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific Company (Oakville, ON, Canada). Double-distilled water (DDW) was used to prepare reagents and chemicals.

4.2.2 Methods

4.2.2.1 Preparation of protein isolates

Soybean and lupin protein isolates were produced according to the method described by Osemwota, Alashi & Aluko, 2021, which was slightly modified as follows. *Lupinus albus* 'Dieta', *Lupinus angustifolius* 'Boregine', and *Glycine max* seeds were milled using RETSCH ZM200 ultracentrifuge (Retsch GmbH, Haan, Germany) mill at 14,000 rpm fitted with a 0.5 mm sieve. The flours produced were defatted by mixing 100 g with 1000 mL acetone and stirred for 2 h. The

solvent phase was discarded, and the process repeated. The pellet obtained after the second mixing with acetone was air-dried in a fume hood overnight. The defatted lupin and soybean flour (100 g) were mixed with DDW (1 L) and adjusted to pH 9 using 1 mol/L NaOH to solubilize the proteins. The mixture was continuously stirred for 1 h and then centrifuged at 5600 x g for 30 min (Beckman Coulter Allegra 6R, Fullerton, CA, USA). The collected supernatant was filtered with a double-layered cheesecloth (grade 90, 40 × 36 thread count per square meters), adjusted to pH 4.5 with 1 mol/L HCl, stirred for 1 h, and then centrifuged again at 5600 x g for 30 min. The resulting precipitate was washed twice with DDW and centrifuged at 5600 x g for 30 min to obtain the final precipitate, which was adjusted to pH 7.0 before freeze-drying to obtain the lupin and soybean protein isolates. The protein content was determined by the modified Lowry method (Markwell, Haas, Bieber, & Tolbert, 1978) using bovine serum albumin as standard.

4.2.2.2 Solubility

The solubility of the protein isolates was assessed following a modified protocol described by (Nwachukwu & Aluko, 2018). In brief, 50 mg of each sample was dispersed in 5 mL of buffers at different pH values (pH 3 – 8). After vortexing and 1 h hydration, the mixture was centrifuged at 5600× g for 30 min. The protein content of each supernatant was determined using the modified Lowry method (Markwell et al., 1978). Additionally, the total protein content of the samples was determined using the same method after dissolving the samples in 1 mol/L NaOH solution. Protein solubility (PS) was then calculated using the formula.

$$PS (\%) = \frac{\text{protein content of the supernatant at a specific pH}}{\text{total protein content of the sample}} \times 100 \quad (1)$$

4.2.2.3 Circular dichroism (CD)

Far- and near-UV CD spectra were measured as described by Osemwota et al., (2021) using a J-815 spectropolarimeter (Jasco Corporation, Tokyo, Japan) at 25 °C. The far-UV CD spectrum was measured with 2 g/L protein dispersed in buffer (pH 3, 5, 7, and 9) at 190-240 nm, using a quartz cuvette with a path length of 0.5 mm. The near-UV spectrum was measured at 250-320 nm with a protein concentration of 4 g/L buffer (pH 3, 5, 7, and 9) using a 1 mm quartz cuvette. The average of five consecutive scans were acquired with simultaneous baseline (buffer) subtraction. Secondary structure fractions were calculated from the far-UV data using the SELCON3 (optimized for 190-240 nm) secondary structure determination algorithm (Miles, Ramalli, & Wallace, 2022) available on the DichroWeb website (<http://dichroweb.cryst.bbk.ac.uk/html/home.shtml>).

4.2.2.4 Oil droplet size distribution of emulsions

Oil-in-water emulsions were prepared according to the method described by Asen & Aluko (2023), with slight modifications. Protein samples of 10, 15, or 20 g/L concentrations were prepared separately in 0.1 mol/L acetate buffer (pH 3), phosphate buffer (pH 5, 7), and Tris buffer (pH 9). Emulsions were prepared by adding 1 mL of refined commercial canola oil to 5 mL of the protein sample, followed by homogenization for 1 min at 20,000 rpm using a Polytron PT 10-35 homogenizer (Kinematica AG, Lucerne, Switzerland) equipped with a 12-mm generator. The emulsion mean surface-weighted oil droplet size ($d_{3,2}$) was determined using a Mastersizer 3000 TM (Malvern Panalytical Ltd., Malvern, UK) with Milli-Q water as the dispersant. Under constant shearing, the emulsion sample was added to approximately 100 mL of water contained in a small-volume wet sample dispersion unit (Hydro 3000 from Malvern) attached to the instrument until obscuration level was attained. Then triplicate measurements of the oil droplet size were acquired and used to calculate the mean value. To determine emulsion stability, the measurement was

repeated after leaving the emulsion undisturbed at room temperature for 30 min and calculated using the following equation.

$$\text{Emulsion stability (\%)} = \frac{\text{Mean oil droplet size at 0 min}}{\text{Mean oil droplet size at 30 min}} \times 100 \quad (2)$$

4.2.2.5 Microstructure characterization

The emulsion microstructure was determined according to the method described by Tian et al., (2020), using a Zeiss laser confocal LSM 700 (CLSM) with a Zeiss Imager M2 microscope (Oberkochen, Germany). The emulsion samples were prepared as described above and stained with an appropriate amount of a mixture of Nile Red (1 g/L, dissolved in ethanol) and fluorescein isothiocyanate (FITC; 1 g/L, dissolved in ethanol). The image detection wavelengths were 561 nm for Nile Red (oil droplets) and 418 nm for FITC (proteins).

4.2.2.6 Flocculation (FI) and coalescence (CI) indices

The FI of the freshly prepared emulsions was determined using a previously described method (Asen & Aluko, 2023). Freshly prepared emulsions were diluted in either DDW or 10 g/L sodium dodecyl sulfate (SDS), and the volume mean droplet diameter ($d_{4,3}$) of droplets was determined at 0 h and 24 h using the Mastersizer 3000 protocol described above. FI (%) was calculated as follows:

$$\text{FI (\%)} = [(d_{4,3} \text{ in water}) / (d_{4,3} \text{ in 10 g/L SDS}) - 1] \times 100 \quad (3)$$

CI was determined after 24 h of quiescent storage at 4 °C as follows:

$$\text{CI (\%)} = [(d_{4,3} (24 \text{ h}) / (d_{4,3} (0 \text{ h}) - 1] \times 100 \quad (4)$$

where $d_{4,3}$ (0 h) and $d_{4,3}$ (24 h) are the $d_{4,3}$ values of droplets in the emulsion at 0 h and 24 h using 10 g/L SDS as the diluent, respectively.

4.2.2.7 Statistical analysis

Triplicate measurements were used to obtain values reported as mean $\pm$ standard deviation. IBM SPSS statistical software version 28 (Chicago, IL, USA) was used to perform the data analysis and the values were tested for statistical significance ($p < 0.05$) using Duncan's multiple comparison test.

4.3 RESULTS AND DISCUSSION

4.3.1 Solubility

The solubility profiles of lupin protein isolates and soy protein isolate (SPI) are shown in Figure 4.1. Notably, the solubility of all isolates exhibited pH dependence, with the lowest solubility occurring around pH 4 to 5, which is consistent with the isoelectric precipitation pH. At the isoelectric point, the net charge of the protein is zero, which minimizes electrostatic repulsion and ionic hydration, leading to protein aggregation and precipitation (Grossmann & McClements, 2023). Legume proteins, including lupin and soybean, tend to aggregate near their isoelectric points, resulting in reduced solubility. Effective emulsification requires proteins to migrate to the oil-water interface and form a film around the oil droplets (Verfaillie, Janssen, Van Royen, & Wouters, 2023). However, this is challenging for poorly soluble proteins. Differences in emulsifying properties among BLPI, WLPI, and SPI can be partly attributed to variations in solubility patterns. The increased solubility of WLPI may be linked to its lower hydrophobic and aromatic amino acid contents, along with higher levels of negatively charged amino acids and non-fiber carbohydrates (Vogelsang-O'Dwyer et al., 2020). Furthermore, as the pH deviates from the isoelectric point, the protein solubility of the isolates increases. This corresponds to the observation of smaller oil droplet sizes at pH values of 3, 7, and 9, where the protein isolates exhibited higher solubility. The results are in agreement with the report of Wang et al. (2023), where pulse electric

field and pH shift method efficiently improved the solubility of SPI by facilitating protein structure unfolding and formation of smaller aggregates.

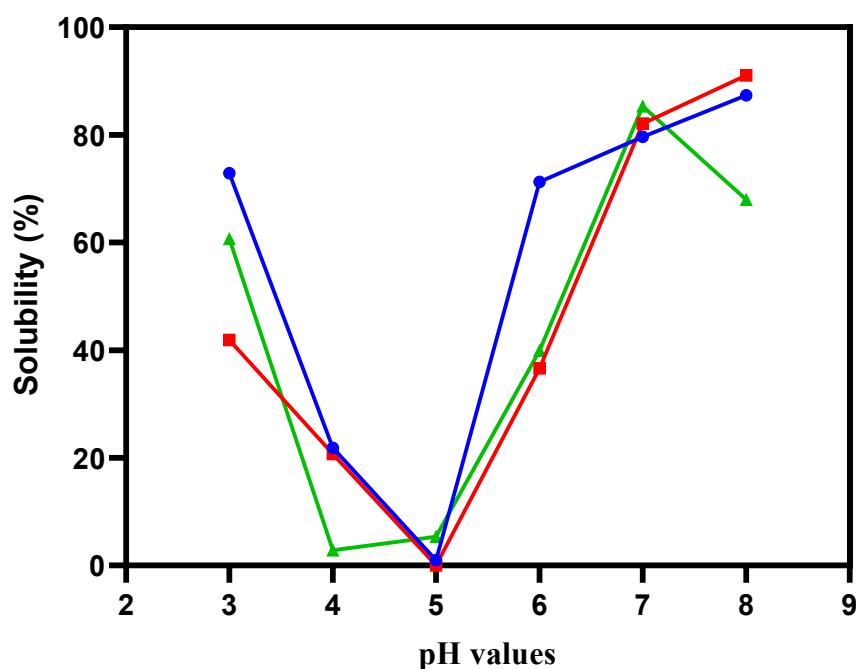


Figure 4.1 Effect of pH on the protein solubility of soybean and lupin protein isolates. ● White lupin protein isolates, ■ Blue lupin protein isolates, ▲ Soybean protein isolates.

4.3.2 Circular dichroism

CD is a spectroscopic technique that relies on the differential absorption of left- and right-circularly polarized light (Tian et al., 2020). CD has been extensively employed in the field of protein structure identification because the structural composition of proteins plays a crucial role in determining functional properties, especially emulsification ability. The far UV results reflect the secondary structures, which show that the α -helical structures were higher for WLPI and SPI at pH 7 and 9 when compared to the BLPI (Table 4.1). In contrast, the BLPI had higher content of the β -sheet structure at all pH values, except pH 9 in comparison to the WLPI and SPI. The BLPI result is similar to the work carried out by Gao et al. (2020) on pea protein isolate and Li et al.

(2021) on soy protein isolate where the β -sheet contents are higher than the α -helix contents. The higher α -helix structure coupled with the lower β -sheet suggests a more flexible protein structure for the WLPI and SPI than the BLPI (Czubinski, 2021). At pH 5, the proteins had higher levels of β -turn fraction, which indicates a more rigid structure when compared to the protein structures at pH 3, 7, and 9. The higher contents of unordered structures present in SPI, WLPI, and BLPI at all pH values in comparison to the other secondary structure fractions indicate highly disordered and less compact protein structures, which may be due to the isoelectric pH-induced changes in the protein ionization state, hydrogen-bonding pattern, and denaturation. This is because at pH values above or below the isoelectric point, some ionizable groups on amino acids, particularly acidic and basic side chains, will become more charged or deprotonated. This change in charge distribution can lead to increased electrostatic interactions between amino acids, which are critical for stabilizing protein structure and preventing denaturation. Therefore, when these interactions are disrupted during isoelectric pH precipitation, the protein could transition into a more disordered state, resulting in an increased random coil content (Aderinola et al., 2020; Jiang, Wang & Xiong, 2018).

The near UV CD spectrum exhibited by each protein is influenced by various factors, including the presence and proximity of aromatic amino acid residues, extent of hydrogen bonding, presence of polar groups, disulfide bonds, and characteristics of chromophores (Miles et al., 2022). At pH 3, both isolates showed similar peak ellipticity at 260, 270, and 290 nm, suggesting phenylalanine, tyrosine, and tryptophan dominance, respectively (Figure 4.2), while SPI peak was predominant at 280 and 290 nm. However, at pH 5, BLPI had a minimal degree of structural organization (high level of denaturation), as evidenced by the spectral values close to zero, whereas WLPI and SPI had slightly increased ellipticity values. At pH 7 and pH 9, both lupin isolates and SPI displayed a

prominent peak at approximately 270 nm, which represents the interaction of buried aromatic amino acid residues in the hydrophobic core with the local environment. The intensity of the peak indicates the presence and stability of the tertiary structure in both lupin isolates and SPI. The peak shift to lower wavelengths at pH 7 and 9 with less broadening of the intensity suggests an increase in surface charges, which pushed the aromatic amino acids away from the hydrophilic environment into the more hydrophobic interior of the protein. In contrast, at pH 3 and 5, the reduced intensity and broad spectra suggest protein structures that are unfolded with increased interactions of the aromatic residues with the hydrophobic environment as previously reported for other proteins (Jiang et al., 2018; Lam & Nickerson, 2015).

Table 4.1 Secondary structure fractions of isoelectric pH-precipitated Soybean protein isolate (SPI), white lupin protein isolate (WLPI), and blue lupin protein isolate (BLPI) at different pH values.

SPI				
pH	3	5	7	9
α -helix (%)	7 ± 3^b	4 ± 1^c	8 ± 3^b	11 ± 4^a
β -sheet (%)	9 ± 1^b	39 ± 3^a	7 ± 3^c	7 ± 3^c
Turns (%)	16 ± 4^b	19 ± 0.3^a	16 ± 4^b	15 ± 1^c
Unordered (%)	67 ± 8^b	39 ± 2^c	69 ± 10^a	67 ± 9^b
WLPI				
pH	3	5	7	9
α -helix (%)	8 ± 3^c	8 ± 3^c	12 ± 3^b	15 ± 0.4^a
β -sheet (%)	6 ± 2^c	13 ± 5^a	7 ± 3^b	8 ± 2^b
Turns (%)	14 ± 5^b	20 ± 3^a	15 ± 5^b	19 ± 0.3^a
Unordered (%)	72 ± 10^a	59 ± 5^c	66 ± 11^b	58 ± 2^c
BLPI				
pH	3	5	7	9
α -helix (%)	6 ± 2^c	5 ± 2^c	8 ± 3^b	10 ± 3^a
β -sheet (%)	21 ± 2^b	34 ± 3^a	11 ± 5^c	6 ± 3^d
Turns (%)	16 ± 3^b	18 ± 0.4^a	17 ± 3^b	14 ± 7^c
Unordered (%)	57 ± 6^c	42 ± 1^d	64 ± 11^b	71 ± 14^a

The data are expressed as mean values ($n=5$) $\pm$ standard deviation. For each row different letters indicate significant differences ($p \leq 0.05$) between the mean values.

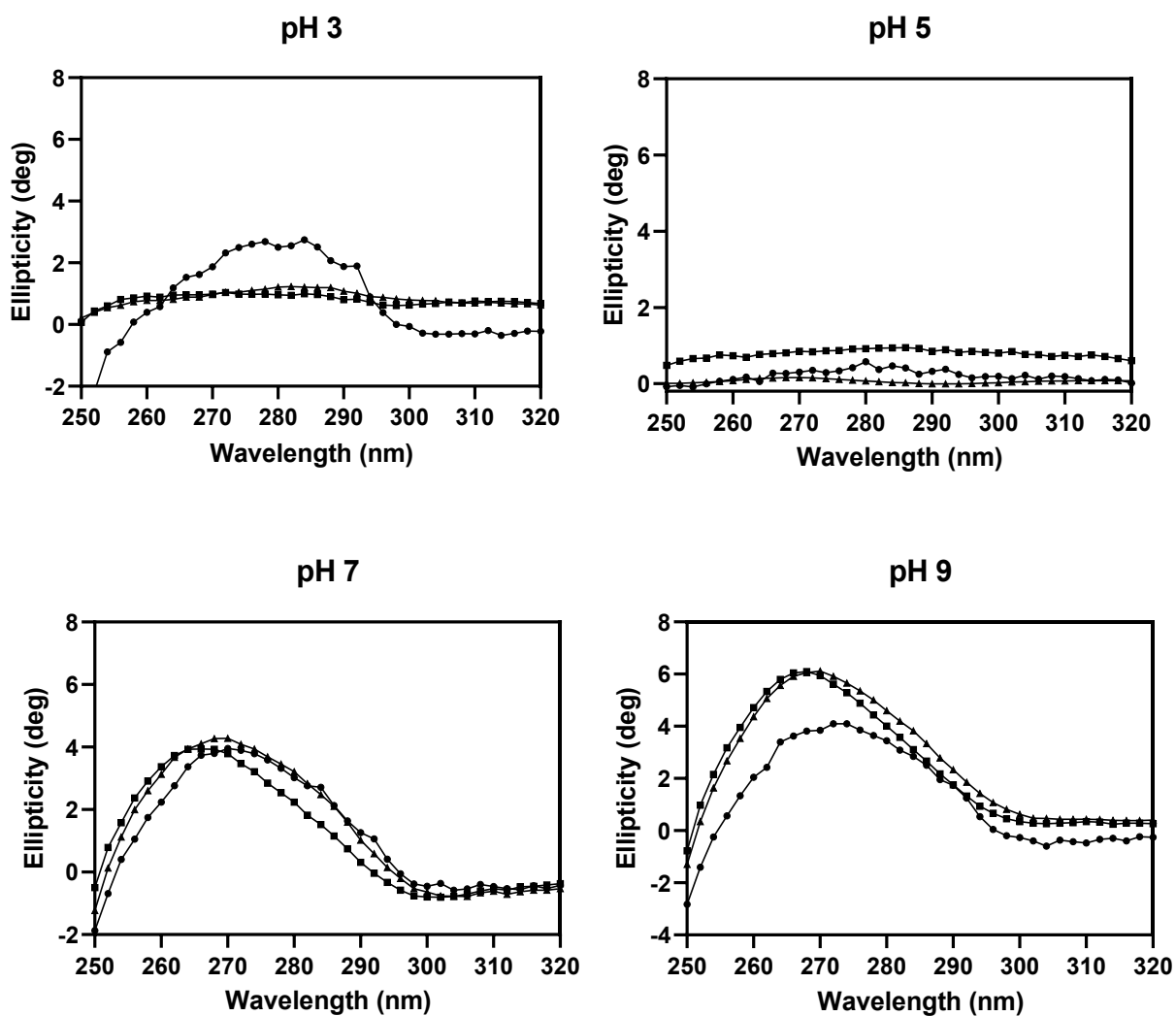


Figure 4.2 Near-UV circular dichroism spectra of isoelectric pH precipitated soybean protein isolate (SPI), white lupin protein isolate (WLPI), and blue lupin protein isolate (BLPI) at different pH values. $\blacksquare$ White lupin protein isolate, $\blacktriangle$ Blue lupin protein isolate, $\bullet$ Soybean protein isolate.

4.3.3 Emulsion formation and stability

The effects of sample concentration and pH on the mean oil droplet size of emulsions formed and stabilized by BLPI, WLPI and SPI are shown in Figure 4.3A. The protein content for SPI (85%), WLPI (91%) and BLPI (83%) together with protein quantity in the continuous phase is crucial for interfacial membrane formation during emulsion production (Wollborn, Michaelis, Ciacchi, & Fritsching, 2022). Therefore, various protein concentrations (10 g/L, 15 g/L, and 20 g/L) were used to represent low, medium, and high protein levels respectively. The mean oil droplet size of emulsions formed using SPI, BLPI, and WLPI was not significantly different across various protein concentrations, suggesting a saturation effect where the presence of additional proteins does not have an impact on the droplet size. These findings align with the notion of increased protein accessibility in the continuous phase, thereby facilitating the development of interfacial membranes crucial for the encapsulation of oil droplets (Burgos-Díaz et al., 2016). The SPI, BLPI and WLPI formed emulsions with larger oil droplet sizes at pH 5, which indicates weaker emulsification ability than at other pH values. This may be because, at pH 5, which is close to the isoelectric point of plant protein where the net charge on the protein surface is zero, the interfacial membrane has low charge density and poor electrostatic repulsions, leading to increased droplet aggregation and formation of large oil droplets. At pH 3, 7, and 9 where the net charge is higher, most emulsions have lower mean oil droplet sizes when compared to pH 5. Overall, pH change had a greater effect on emulsion formation than did protein concentration, indicating the importance of environmental modification. These findings are consistent with those of recent reports on pea protein isolate (Guldiken, Saffon, Nickerson, & Ghosh, 2023), where pea protein isolates (PPI) alone produced stable emulsions at pH 8, but unstable and aggregated emulsions at pH 4.5. When PPI was complexed with soluble pectin, the mixture produced stable emulsions at

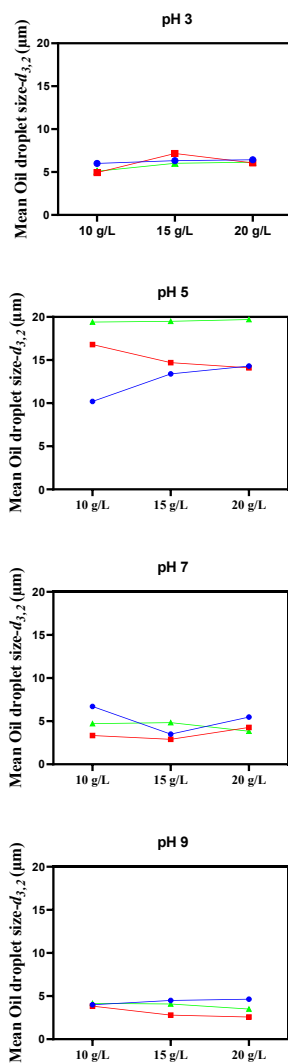
pH 4.5 with smaller droplet size and lower aggregation, with significant implications for the development of plant protein-based mildly acidic beverages.

Liu & Tang, 2014 corroborate the influence of protein concentration on the emulsifying properties of SPI. They concluded that increasing the protein concentration resulted in the formation of emulsions with smaller droplet sizes. This relationship between protein concentration and droplet size can be attributed to the enhanced surface activity and coverage of the oil-water interface by SPI molecules at higher concentrations. As more proteins become available in the continuous phase, they adsorb more effectively to the surface of oil droplets, reducing the interfacial tension and promoting the formation of smaller and more stable droplets. This phenomenon is consistent with findings from other studies on plant proteins, which suggest that an optimal protein concentration facilitates the formation of a cohesive and continuous interfacial film crucial for preventing droplet coalescence (Tang, 2017). Additionally, heated SPI with large particle sizes effectively stabilizes the oil droplets within an emulsion because it provides a robust interfacial layer that resists coalescence. Homogenization conditions and ionic strength can enhance the adsorption of SPI at the oil-water interface, thereby improving the stability of the emulsion (Cui et al., 2014).

The emulsion stability was estimated by measuring changes in the mean oil droplet size after a 30-min storage at room temperature (Osemwota et al., 2021). As shown in Figure 4.3B, WLPI at 20 g/L displayed the highest emulsion stability (100%) at all pH values. In contrast, the emulsion stability of BLPI at pH 9 (50%) was lower than that at all other pH values and also decreased with increase in protein concentration. SPI displayed fairly stable emulsion at all pH values. This may be a result of the well-balanced distribution of hydrophilic and hydrophobic amino acids, enabling

them to interact effectively with both the oil and aqueous phases (Tang, 2017). SPI proteins can also quickly adsorb to oil-water interfaces, forming a protective layer around the droplets. This rapid adsorption is important for creating stable emulsions and for preventing droplet coalescence immediately after emulsification. The structural flexibility of soybean proteins allows them to unfold and reorient at the oil-water interface across various pH levels, which is crucial for maintaining stability under different conditions (Tang, 2017). With the exception of WLPI at pH 7 and BLPI at pH 9, emulsion stability was enhanced at higher protein concentrations, which suggests positive influence of a strong interfacial film and increased viscosity of the continuous aqueous phase. The results suggest that the BLPI had a higher charge density at pH 9, which prevented the formation of a viscous continuous phase and a strong interfacial membrane. Bulk-phase viscosity also contributes to stability independently of the interfacial film. By Stokes' law, the creaming rate is inversely proportional to the continuous-phase viscosity. At pH 9, BLPI's strong net negative charge promotes electrostatic repulsion, keeping the protein molecularly dispersed rather than forming a viscous network; the resulting low-viscosity phase offers little resistance to creaming. This also explains the decline in stability with increasing protein concentration, since excess non-adsorbed protein is more likely to promote depletion flocculation than thickening. Direct rheological measurement of the continuous phase across pH and concentration would confirm this mechanism. Except for the relatively low emulsion stability of BLPI at pH 3 and pH 9, both lupin isolates exhibited high emulsion stability (over 80%), indicating their potential use as ingredients in the formulation of food emulsions where short-term stability is required.

A



B

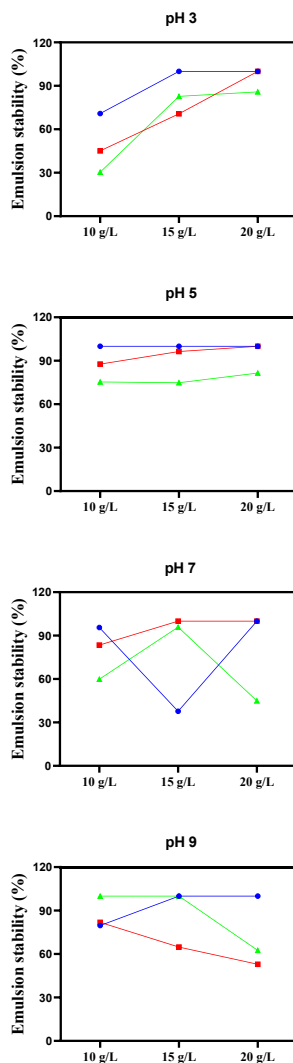


Figure 4.3 (A) Effect of pH and protein concentration on the mean oil droplet sizes ($d_{3,2}$) of soybean protein isolate (SPI), white lupin protein isolate (WLPI), and blue lupin protein isolate (BLPI) emulsions. (B). Effect of pH and protein concentration on the stability of soybean protein isolate (SPI), white lupin protein isolate (WLPI), and blue lupin protein isolate (BLPI) emulsions.

● WLPI, ■ BLPI, ▲ SPI.

4.3.4 Emulsion microstructure

CLSM images of freshly prepared emulsions of both isolates at pH 3, 5, 7, and 9 (10 g/L protein) are shown in Figure 4.4. At different pH values, the emulsions had distinct variations in the oil droplet sizes and proteins adsorbed to the interfacial layer, which is an indication of suitable emulsification properties. For all the isolates, the emulsions prepared at pH 7 and 9 showed a well-defined protein interfacial film around the oil droplets dispersed in the continuous phase. The spherical oil droplets which were less than 9 μm were evenly distributed, indicating an efficient adsorption at the interfacial layer. This is a good indication of the superior emulsion capacity observed at pH 7 and 9 for both the isolates and is consistent with the smaller oil droplet sizes as shown in Figure 4.3A. The content of α -helix fraction is also higher at pH 7 and 9, hence could have contributed to the better emulsion forming ability (smaller oil droplet sizes) when compared to pH 5. At pH 3, the WLPI and SPI formed emulsions with smaller oil droplets ($< 6 \mu\text{m}$) than the BLPI, which is consistent with the mean oil droplet size for 10 g/L (Fig. 3A) and may be due to differences in secondary structure fractions. This is because WLPI and SPI had more of the flexible α -helix and less of the rigid β -sheet and β -turns (Table 4.1), hence better emulsion forming ability when compared to the BLPI. For the emulsions formed at pH 5 for both isolates, the oil droplets were noticeably bigger and more prominent, which is an indication of a rigid protein conformation and hence poor emulsification ability. At pH 5, the WLPI had lower content of the rigid β -sheet and β -turns, but higher level of the flexible α -helix, which could have contributed to the lower number of big oil droplets when compared to the BLPI. Similarly, both SPI and WLPI had a more native (compact) tertiary structure at pH 5 (Figure 4.2), which may have contributed to the better emulsion forming ability when compared to the BLPI that showed a lack of well-defined structure. The results at pH 5 also correlate with the mean oil droplet size where at 10 g/L, the WLPI had a

mean value of approx. 10 μm in contrast to the 16 μm for the BLPI (Figure 4.2A). Overall, the results suggest that the secondary and tertiary structure attributes are important contributors to the emulsification properties of lupin and soybean proteins.

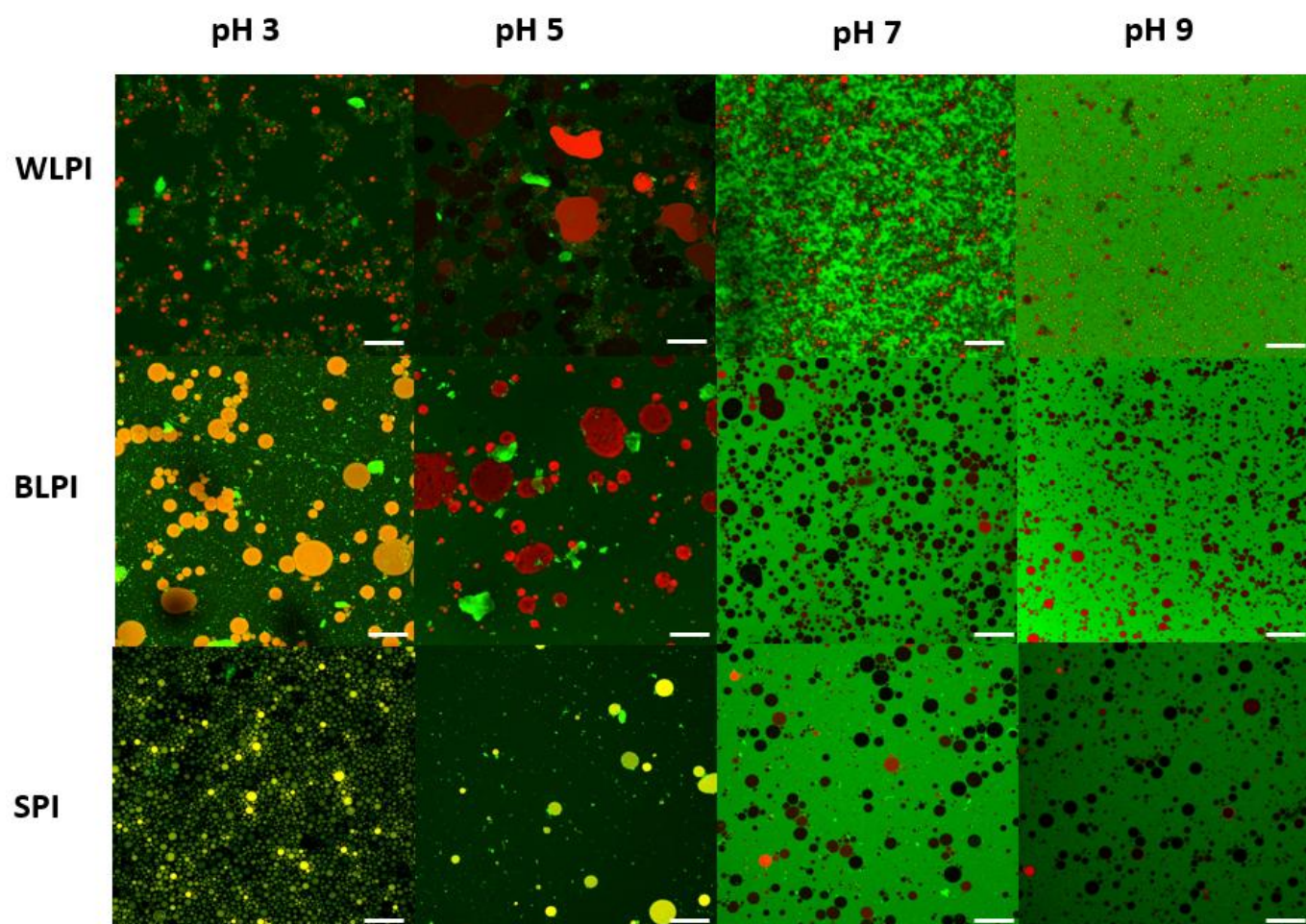


Figure 4.4 Confocal laser scanning microscopy (CLSM) images of freshly prepared soybean protein isolate (SPI), white lupin protein isolate (WLPI) and blue lupin protein isolate (BLPI) emulsions. The scale bars within figures represent 40 μm in length.

4.3.5 Flocculation and coalescence

The flocculation (FI) and coalescence (CI) indices are important parameters that can be used to gauge the stability of emulsions (Vogelsang-O'Dwyer, Sahin, Zannini, & Arendt, 2022). Coalescence is the merging of two or more droplets to form a single, larger droplet, whereas flocs form when two or more emulsified oil droplets adhere to form aggregates without losing their individual shapes (Asen & Aluko, 2023). Consequently, a higher level of flocculation indicates a lower level of emulsion stability. The FI of freshly prepared (0 h) and stored emulsions (24 h) was calculated as the relative change in $d_{4,3}$ values (Figure 4.5). pH had a significant effect on the FI of freshly prepared emulsions (0 h). For both lupin protein isolates and SPI, the emulsions prepared at pH 5 had the highest tendency to form flocs (higher FI), whereas most of the emulsions prepared at pH 7 existed in the non-flocculated state. This corresponds to the CLSM data (Figure 4.3), where aggregated flocs were visible at pH 5, which may be attributed to reduced interfacial charge and hence increased hydrophobic interactions between the droplets to stabilize themselves in a hydrophilic environment. The results are also consistent with the bigger oil droplet sizes of the emulsions at pH 5 when compared to pH 3, 7, and 9 (Figure 4.2). The FI of the emulsions prepared at pH 7 indicates that this pH is highly suitable for preparing lupin and soybean protein-stabilized emulsions with homogenous and small oil droplet sizes. The stability of the flocs declined after storage for 24 h, with the FI increasing at all protein concentrations for both protein isolates at pH 3, 5, and 7. However, emulsions prepared at pH 9 (24 h) for both lupin isolates and SPI maintained a low FI index, indicating better stability against flocculation, which may be due to increased net charge of the interfacial membrane that reduced attractive interactions between the oil droplets.

Coalescence is mainly dependent on the integrity of the viscoelastic film surrounding the oil droplets (Tian et al., 2020). The results from Figure 4.6 showed that droplet coalescence occurred

mostly at pH 9, which is indicative of a thin interfacial membrane. This could be because as the pH increases above the isoelectric point, protein side groups ionize and there is increased charged density, which prevents formation of adequate protein-protein interactions needed to form a strong interfacial membrane. This is supported by the results showing increased protein concentration at pH 9 led to reduced CI, which suggests that the addition of more protein molecules helped stabilize the interfacial membrane against disruption. None of the emulsions formed at pH 7 showed evidence of oil droplet coalescence, which is consistent with the lowest rate of flocculation as shown in Figure 4.5A. Overall, stability was more dependent on the flocculation rate and less on the oil droplet coalescence, which indicates that most of the emulsions had strong interfacial membranes that were resistant to breakage, but reduced charge density may have enhanced oil droplet aggregation.

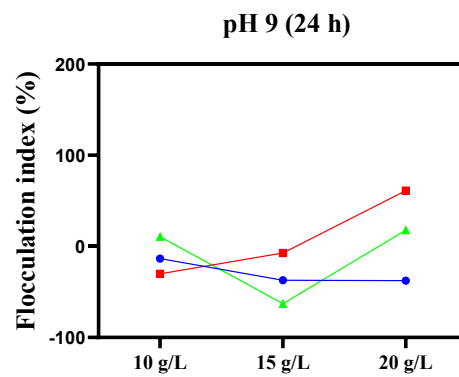
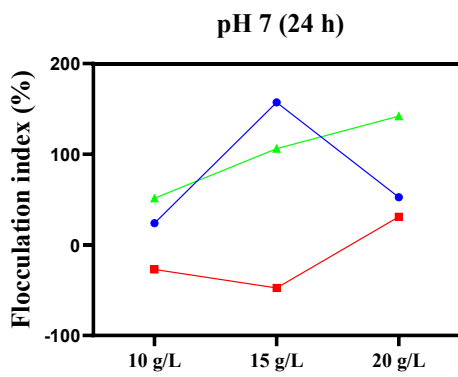
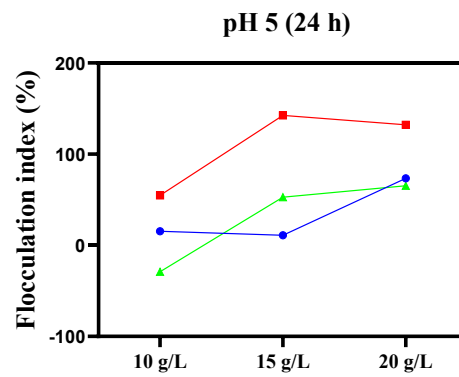
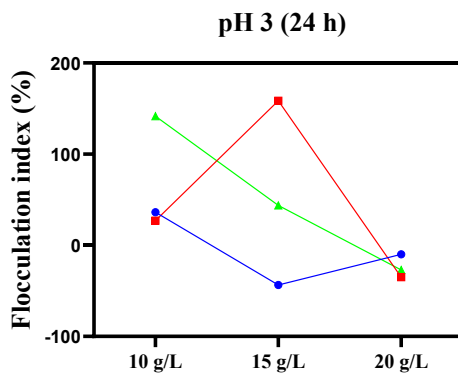
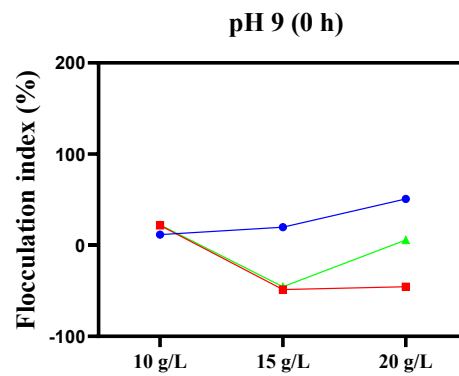
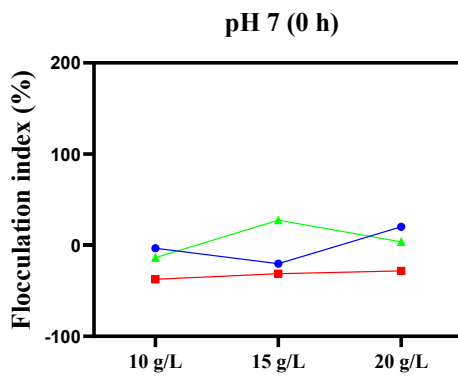
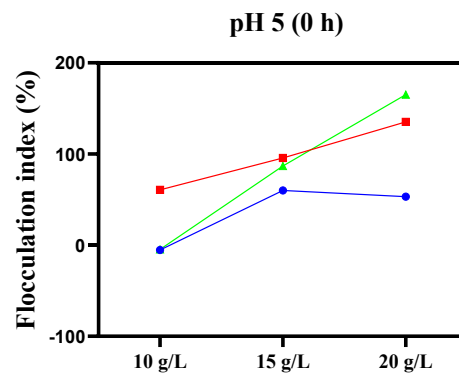
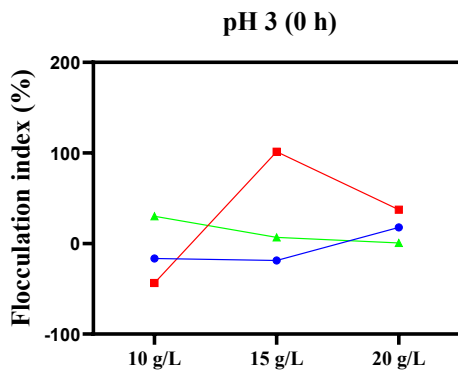


Figure 4.5 The flocculation index (FI) of freshly prepared (0 h) and stored emulsions (24 h) of soybean protein isolate (SPI), white lupin protein isolate (WLPI), and blue lupin protein isolate (BLPI) at different pH and protein concentrations. ● WLPI, ■ BLPI, ▲ SPI.

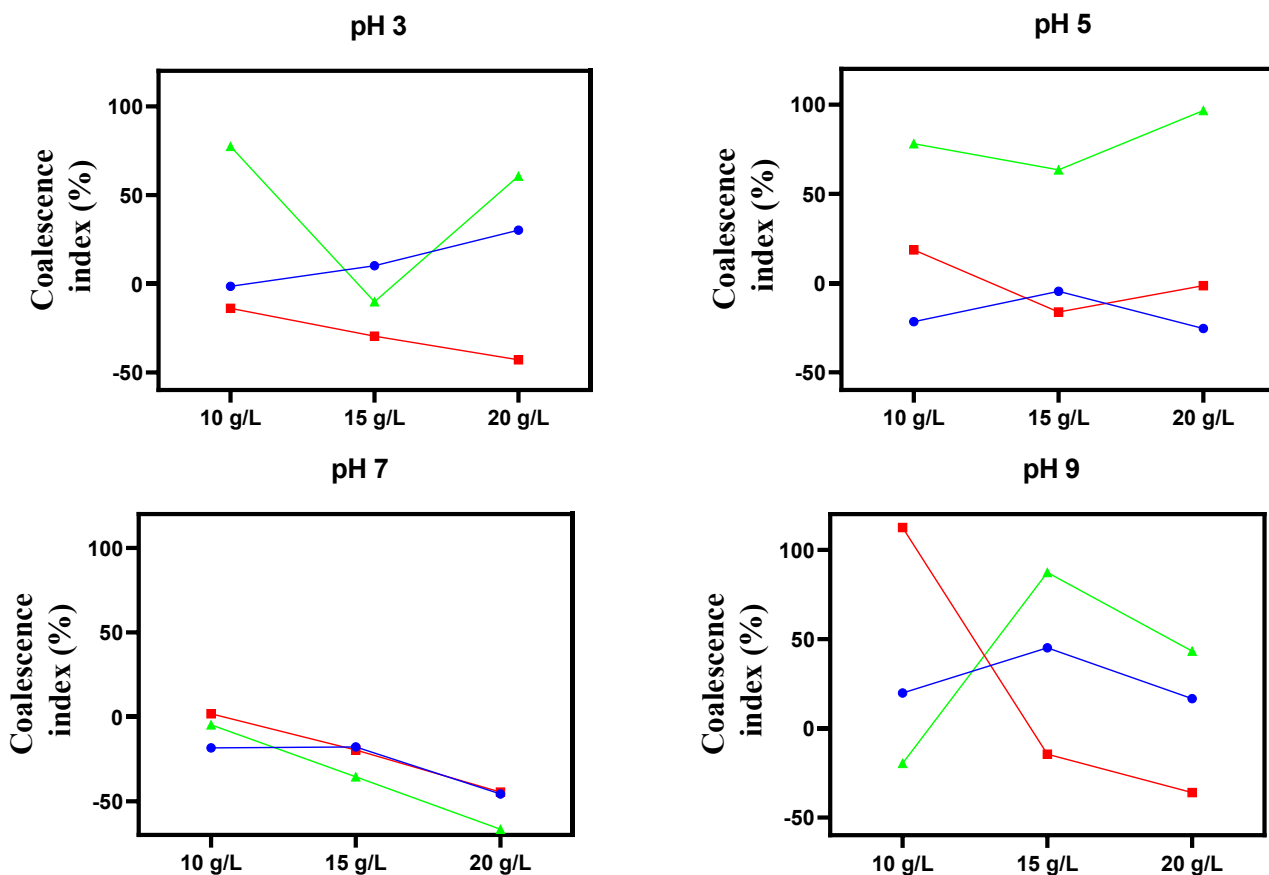


Figure 4.6 Coalescence index of soybean protein isolate (SPI), white lupin protein isolate (WLPI), and blue lupin protein isolate (BLPI) emulsions prepared at different pH and protein concentrations. ● WLPI, ■ BLPI, ▲ SPI.

4.4 CONCLUSIONS

The emulsifying properties of SPI, WLPI, and BLPI varied considerably upon treatment at different pH values and protein concentrations, with improved emulsifying properties observed at pH 7 and 9. The secondary and tertiary structure of the protein isolates plays a critical role in the oil droplet size and emulsion stability. The higher presence of α -helical structures correlated with

the formation of smaller oil droplet sizes and a higher content of β -sheets and β -turns led to the formation of larger oil droplet sizes and poor emulsification properties. Also, droplet coalescence contributed less to emulsion stability than bridging flocculation. The observed superior emulsifying properties of BLPI at neutral pH, with lower flocculation (FI) and coalescence indices (CI) after 24 hours of storage, highlight its potential as an effective plant-based emulsifier. This insight is significant given the increasing demand for plant-based ingredients due to dietary preferences and sustainability concerns. Although the emulsifying properties were primarily examined under controlled pH conditions, these may not fully capture the complex environments encountered in practical applications. Therefore, in our subsequent research, we will examine the impact of other food matrix components such as salts, sugars, and fibers on the emulsifying properties of these protein isolates and also investigate the interactions of lupin protein emulsions with different food emulsion systems.

AUTHORSHIP

R.E.A Conceived, designed, supervised, reviewed, and edited the manuscript's final draft, S.C. carried out the research, analyzed the data, and wrote the first draft of the manuscript.

CRedit AUTHORSHIP CONTRIBUTION

Stanley Chukwuejim: Writing— original draft, Methodology, Investigation, Formal analysis.

Rotimi E. Aluko: Writing— review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY

Data will be made available on request

ACKNOWLEDGMENTS

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TRANSITION STATEMENT

Chapter 4 (Manuscript 3) demonstrated that the emulsifying properties of blue lupin, white lupin, and soybean protein isolates are strongly influenced by pH, protein concentration, and secondary structure composition. The study showed that all three isolates formed smaller and more stable oil droplets at pH 7 and 9, conditions associated with higher α -helical content, whereas emulsification was poorest near the isoelectric point (pH 5), where increased β -sheet content and pronounced bridging flocculation were observed. BLPI exhibited superior emulsifying performance at neutral pH with lower flocculation and coalescence indices after 24 h of storage, highlighting its potential as an effective plant-based emulsifier. These results confirmed that lupin protein isolates can achieve emulsification performance comparable to that of soybean protein isolates under appropriate conditions. However, the studies in Chapters 3 and 4 examined lupin proteins exclusively at the whole-isolate level, which represents a complex mixture of albumin, globulin, and glutelin fractions with different structural features and surface properties. The functional behavior observed for the isolates reflects the net contribution of these individual components, potentially masking the superior performance of specific fractions or the limiting effect of others. To resolve these contributions and identify which protein classes are responsible for the observed functional differences between species, Chapter 5 (Manuscript 4) fractionated both white and blue lupin protein isolates into their albumin, globulin, and glutelin constituents followed by systematic evaluation of the conformational state and functional performance of each fraction. This fraction-level analysis provides the mechanistic insight needed to explain the isolate-level observations reported in the previous chapters.

CHAPTER FIVE

MANUSCRIPT FOUR

LUPIN SPECIES AND PROTEIN FRACTION TYPE AFFECT THE CONFORMATIONAL STATE AND FUNCTIONAL PERFORMANCE OF THE PROTEINS

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ABSTRACT

This study investigated the effects of lupin species, pH, and protein concentration on the structural and functional properties of albumin, globulin, and glutelin protein fractions isolated from white (*Lupinus albus*) and blue (*Lupinus angustifolius*) lupin seeds. The globulin and glutelin fractions exhibited characteristic pH-dependent U-shaped solubility curves, with minimum solubility near at pH 5. Amino acid analysis revealed that white lupin globulin (WLGlo) was the only fraction not limited in sulfur-containing amino acids (methionine + cysteine: 107% amino acid score). Blue lupin glutelin (BLGlu) demonstrated high surface hydrophobicity (3924.1), *in vitro* protein digestibility (92.44%), and the highest intrinsic fluorescence intensity at pH 3, indicating a conserved but enzyme accessible protein conformation. Far-UV circular dichroism analysis revealed that white lupin albumin (WLA1b) possessed the highest α -helix content at pH 7 (23%) and pH 9 (18%), reflecting a more flexible conformation. Emulsions stabilized by WLGlo at pH 3, 7, and 9 had smaller oil droplet sizes ($\sim$ 4-7 μ m) than most of the other protein fractions. All fractions displayed excellent foaming properties, with stability exceeding 85%, and glutelin fractions showing particularly good (50-60%) foaming capacity at pH 5. These findings establish clear structure-function relationships and demonstrate the potential of lupin protein fractions as high-performance ingredients for diverse food applications, with each fraction offering specific advantages based on nutritional quality, structural characteristics, and functional properties.

Keywords

Lupin, protein fraction, surface hydrophobicity, fluorescence intensity, circular dichroism, functional properties

5.1 INTRODUCTION

Lupin seeds (*Lupinus spp*) are one of the most promising yet underutilized legume crops, with exceptional nutritional potential, containing 28-48% protein, high fiber content, and rich in bioactive compounds (Chukwuejim et al., 2023; van de Noort, 2024). Despite their high protein content and sustainable cultivation characteristics, lupin proteins remain significantly underutilized in food applications, particularly when compared to extensively studied legumes, such as soy, pea, and chickpea (Deng & Kim, 2024; Patil et al., 2024; Tulbek et al., 2024). Recent investigations of various legumes have revealed substantial functional diversity among protein fractions, with studies on faba bean, chickpea, and pea proteins demonstrating enhanced emulsification properties through targeted fractionation (Chang et al., 2022; Stone et al., 2024). Research on lentil and mung bean proteins has also revealed distinct physicochemical properties that correlate with specific functional applications in food systems (Dias et al., 2024; Feng et al., 2024; Shevkani et al., 2024). The growing global demand for sustainable protein sources, coupled with increasing consumer awareness of plant-based nutrition, has intensified the research focus on optimizing the extraction and characterization of legume proteins. However, despite these advances in legume protein research, lupin proteins have received disproportionately limited attention, representing a significant opportunity to expand the plant protein ingredient portfolio. The nitrogen-fixing capacity of lupin crops, combined with their ability to thrive in marginal soils, positions them as environmentally sustainable protein sources that could significantly contribute to addressing global protein security challenges while reducing environmental impacts (Lucas et al., 2015). Furthermore, the unique amino acid composition of lupin protein fractions, particularly their high lysine content and balanced essential amino acid profile, positions them as valuable ingredients for nutritionally enhanced food products targeting specific demographic groups, including children and the elderly (Vogelsang-O'Dwyer et al., 2020).

Legume seed proteins are classified into four major fractions based on their solubility characteristics: albumins (water-soluble), globulins (salt-soluble), prolamins (alcohol-soluble), and glutelins (alkali-soluble), with globulins comprising 80-90% of total seed proteins for lupin seeds (Duranti et al., 2008). The globulin fraction consists primarily of two storage proteins: α -conglutin (legumin-type, 11S), which is characterized by high thermal stability and contributes significantly to the gelation properties of lupin protein (Muranyi et al., 2016), and β -conglutin (vicilin-type, 7S), a trimeric protein with a molecular weight of approximately 150-260 kDa. This fraction is known for its excellent emulsifying properties and its role in foam formation and stability (Shrestha et al., 2021). Although albumin fractions represent only 10-15% of total proteins, they contain bioactive compounds, including enzyme inhibitors and lectins, that contribute to the nutritional and functional properties of isolated plant proteins (Boukid & Pasqualone, 2022). For example, γ -conglutin has garnered interest because of its potential bioactive properties, including hypoglycemic effects (Tapadia et al., 2021). Sequential extraction protocols have been developed to isolate these fractions, typically involving water extraction for albumins, followed by salt solutions (0.5-1.0 M NaCl) for globulins, alcohol-based solvents (70-80% ethanol) for prolamins, and alkaline solutions (0.05 M NaOH) for glutelins (Adebiyi & Aluko, 2011). The functionality of these protein fractions is intrinsically dependent on their molecular architecture, including charge distribution, molecular size, three-dimensional structure, and folding patterns, all of which are influenced by environmental conditions, particularly the pH (Devkota et al., 2024). At pH values near their isoelectric points, protein molecules exhibit minimal net charge, leading to reduced electrostatic repulsion, increased protein-protein interactions, and consequently diminished solubility and altered functional properties, such as emulsification and foaming capacity (Albe-Slabi et al., 2022). Conversely, at extreme pH values (pH < 3 or pH > 8),

proteins carry significant net charges that enhance electrostatic repulsion, which promotes protein unfolding, and improve solubility, while potentially compromising structural integrity (Nahimana et al., 2024). Processing conditions, including ionic strength, temperature, and mechanical treatments, further modulate protein conformation through effects on hydrophobic interactions, hydrogen bonding, and disulfide bridge formation, ultimately determining functional performance in food applications (Lo et al., 2021). Studies on the structural, physicochemical, and functional properties of different legumes, such as pea (Adebiyi & Aluko), chickpea (Perovic et al., 2025; Ye et al., 2024), lentil (Osemwota et al., 2022), mungbean (Tang & Sun, 2010) and fava bean (Oluwajuyitan & Aluko, 2025) have been carried out, demonstrating a growing interest in legumes as sustainable protein sources for food product development and nutritional enhancement.

Despite the growing interest in lupin proteins, significant knowledge gaps exist regarding the comprehensive characterization and comparative analysis of individual protein fractions from different lupin species, particularly the systematic evaluation of structural-functional relationships across albumin, globulin, and glutelin fractions from blue (*Lupinus angustifolius*) and white (*Lupinus albus*) lupin species. While previous studies have focused primarily on whole-protein isolates, limited research has examined the complete protein fraction profile and species-specific differences in functional properties. Information on how the structural characteristics and functional properties of albumin, globulin, and glutelin fractions differ between blue and white lupin species and the implications for targeted food applications is scarce. The significance of this study lies in its potential to provide a foundation for optimizing lupin protein utilization in food systems, enabling the targeted selection of specific fractions for particular applications while maximizing the functional potential of underutilized lupin species. Understanding these species-specific and fraction-specific differences could revolutionize lupin protein applications, potentially

unlocking new markets for sustainable plant proteins and contributing to enhanced food security through diversified protein sources. We hypothesized that blue and white lupin species exhibit distinct protein fraction profiles with unique structural characteristics that translate into specific functional advantages, a characteristic that varies significantly between species. The objective of this study was to determine the effect of lupin species, pH, and protein concentration on the structure and function of lupin albumin, globulin, and glutelin fractions, which could provide a scientific rationale for their selection in specific food system applications, ultimately contributing to enhanced utilization of lupin proteins in sustainable food production systems.

5.2 MATERIALS AND METHODS

5.2.1 Materials

The Lupin Platform Inc. (Calgary, AB, Canada) donated blue (*Lupinus angustifolius* ‘Boregine’) and white (*Lupinus albus* ‘Dieta’) lupin seeds. Glycine, 1-Anilino-8-naphthalenesulfonate (ANS), 5,5'-Dithiobis-2-nitrobenzoic acid, Sodium dodecyl sulfate, Tris(hydroxymethyl)aminomethane, Urea, Sodium chloride, Hydrochloric acid, and Sodium hydroxide were sourced from Sigma-Aldrich (St. Louis, MO, USA). Potassium dihydrogen phosphate, Disodium hydrogen phosphate, and Ethylenediaminetetraacetic acid were sourced from Fisher Scientific Company (Oakville, ON, Canada). Mini-PROTEAN precast TGX gels (4-15%) were sourced from Bio-Rad Laboratories (Hercules, CA, USA). All other reagents and chemicals utilized in this study were of high-purity analytical grade. The reagents and chemicals were prepared using double-distilled water (DDW).

5.2.2 Methods

5.2.2.1 Protein extraction and fractionation

Ground lupin flours were defatted with acetone using a previously described method Chukwuejim & Aluko (2025). Sequential extraction (Figure S1) began with 100 g of defatted lupin flour, which

was subjected to salt extraction using a 2% NaCl solution (1:10 w/v) for 4 h at room temperature with continuous stirring. The mixture was centrifuged at $5600 \times g$ for 30 min at 4 °C. The supernatant containing salt-soluble proteins was collected, and the pellet (Pellet-1) was retained for subsequent extraction steps. The salt-soluble extract was dialyzed against distilled water using a 6-8 kDa molecular weight cut-off membrane for 48 h at 4°C, with multiple water changes. Following dialysis, the solution was centrifuged at $5600 \times g$ for 30 min to separate the water-soluble (WS) fraction (supernatant) from the salt-soluble (SS) fraction (precipitate). Both fractions were collected separately and freeze-dried as the albumin and globulin, respectively. Pellet-1 underwent extraction with 70% ethanol (1:10 w/v) at room temperature for 4 h with continuous stirring. The mixture was centrifuged at $5600 \times g$ for 30 min, and the supernatant containing ethanol-soluble proteins was collected while the pellet (Pellet-2) was retained for the final extraction step. The ethanol-soluble extract was dialyzed against distilled water for 48 h at 4°C, after which the dialyzed solution was freeze-dried to obtain the ethanol-soluble (ES) fraction (prolamin). Pellet-2 was subjected to alkaline extraction using 0.05 M NaOH (1:10 w/v) at room temperature for 30 min with continuous stirring. The mixture was centrifuged at $5600 \times g$ for 30 min, the supernatant containing alkaline-soluble proteins was collected, and the residue was discarded. The alkaline-soluble extract was dialyzed against distilled water for 48 h at 4°C, and the dialyzed solution was freeze-dried to obtain the alkaline-soluble (AS) fraction (glutelin). The protein content in each freeze-dried fraction was determined using the modified Lowry method (Markwell et al., 1978). The gross yield was calculated using the following formula (equation 1).

$$\text{Gross yield (\%)} = \frac{\text{Weight of dried protein fractions (g)}}{\text{Weight of defatted lupin flour (g)}} \times 100 \quad (1)$$

The protein yield (recovery) was calculated using the following formula (equation 2).

Protein Yield (%)

$$= \frac{\text{weight of dried protein fraction (g)} \times \text{fraction's protein content (\%)}}{\text{Weight of defatted lupin flour (g)} \times \text{flour's protein content (\%)}} \times 100 \quad (2)$$

5.2.2.2 Amino acid composition

The amino acid composition of lupin protein fractions was analyzed by high-performance liquid chromatography (HPLC) using a Shimadzu Nexera X2 system (Shimadzu Corp., Kyoto, Japan) equipped with a Pico-Tag column. Prior to analysis, samples were hydrolyzed in 6 M hydrochloric acid for 24 h (White et al., 1986). Sulfur-containing amino acids (cysteine and methionine) were quantified following performic acid oxidation (Spindler et al., 1984), whereas tryptophan was determined separately after alkaline hydrolysis (Landry & Delhaye, 1994). Post-hydrolysis, amino acids were derivatized by combining 70 µL of AccQ-Tag buffer, 10 µL of sample or standard, and 20 µL of AccQ-Tag reagent, followed by incubation at 55 °C for 10 min. Standard amino acids were separated on an AccQ-Tag Ultra column attached to a Nexera X2 UPLC system (Shimadzu Corp., Tokyo, Japan) using a linear gradient of AccQ-Tag buffers A and B over 17 min at a flow rate of 0.7 mL/min and a column temperature of 51 °C, with UV detection at 260 nm. Cysteine and methionine were analyzed using fluorescence detection under optimized conditions at 40 °C and 60 °C, respectively, with a flow rate of 0.4 mL/min and excitation/emission wavelengths of 266/473 nm. Tryptophan analysis was performed using a C18 column (Phenomenex, Torrance, CA, USA) attached to the Nexera X2 UPLC system following isocratic elution at 1.0 mL/min and 28 °C for 34 min with a mobile phase comprising 0.3% acetic acid, 0.05% 1,1,1-trichloro-2-methyl-2-propanol, and 5% methanol, adjusted to pH 5.0 using ethanolamine; fluorescence detection was conducted at excitation and emission wavelengths of 280 and 356 nm, respectively. Amino acid scores were calculated in accordance with the FAO/WHO (2013) reference pattern for

older children, adolescents, and adults (Sa et al., 2024). The amino acid score is calculated following equation (3):

$$\text{Amino acid score (\%)} = \frac{\text{mg of amino acid per g of test protein}}{\text{mg of same amino acid per g of reference protein}} \times 100 \quad (3)$$

5.2.2.3 Gel electrophoresis

The polypeptide profile of lupin protein fractions was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), according to the method described by Aderinola et al. (2020). A stock solution of lupin protein fractions was prepared at a concentration of 10 mg/mL. This solution was then diluted to achieve a final concentration of 1 mg/mL for analysis. The protein fractions were mixed with sample buffer composed of Tris-HCl (pH 6.8), 10% SDS, glycerol, double-distilled water, and 1% bromophenol blue. Two sets of samples were prepared, one with β -mercaptoethanol (reducing conditions) and the other without β -mercaptoethanol (non-reducing conditions). The mixtures were subjected to heat treatment at 100°C for 5 min, followed by cooling to room temperature. Subsequently, 10 μ L aliquots of each sample were loaded onto Bio-Rad Mini-Protein precast gels. A protein standard marker ranging from 10 to 200 kDa was used as the molecular weight reference. After electrophoresis, the gels underwent a 2-h staining process using Coomassie brilliant blue. Destaining was performed using a solution of 50% methanol and 10% acetic acid. The resulting protein band patterns were visualized and documented using a Biorad ChemiDoc™ imaging system (Bio-Rad Laboratories Inc., CA, USA).

5.2.2.4 Surface hydrophobicity

Surface hydrophobicity (So) was determined using the fluorescent probe 8-Anilino-naphthalene-1-sulfonic acid (ANS), following the method of Kato and Nakai (1980), which was slightly modified. Briefly, a stock protein solution (10 mg/mL) was serially diluted to concentrations ranging from 50 to 250 μ g/mL using 0.01 M phosphate buffer (pH 7.0). A 200 μ L aliquot of each concentration

was combined with 20 μ L of ANS solution (8.0 mM in 0.01 M phosphate buffer pH 7). The fluorescence intensity (FI) was measured using a Jasco FP-6300 spectrofluorometer (Jasco Inc., Tokyo, Japan) with excitation and emission wavelengths of 390 and 470 nm, respectively. The S_o value was derived from the slope of the linear regression plot of FI versus protein concentration.

5.2.2.5 Differential scanning calorimetry

Thermal analysis was performed using a TA Q100-DSC thermal analyzer (TA Instruments, New Castle, DE, USA). Each protein fraction (10 mg) was placed into aluminum pans and then hermetically sealed. Thermal scanning was conducted from 40°C to 140°C at a heating rate of 10°C/min. An empty sealed pan served as the reference. All measurements were performed in duplicate to ensure repeatability. The instrument's software (Universal Analysis 2000, Version 4.5) was used to determine key thermal parameters, including the onset temperature (T_o), denaturation temperature (T_d), and enthalpy of denaturation (ΔH).

5.2.2.6 Sulfhydryl group content

Sulfhydryl (SH) and disulfide (SS) groups were quantified following the protocol outlined by Asen and Aluko (2022). Protein fractions were dispersed in Tris-Gly buffer (0.086 M Tris, 0.09 M glycine, 0.004 M EDTA, pH 8.0) containing 8 M urea to achieve a 7.5 mg/mL concentration. The dispersion was stirred overnight at ambient temperature. For free SH content analysis, a 1 mL aliquot of the stirred protein dispersion was combined with 4 mL of Tris-Gly buffer and 0.05 mL of Ellman's reagent. The mixture was incubated for 20 min at 25°C, after which the absorbance was measured spectrophotometrically at 412 nm using the Ultrospec 4300 Pro UV–Visible spectrophotometer (Amersham Biosciences, London, UK). Determining the total SH content (encompassing free SH and reduced SS) involved mixing 1 mL of the stirred protein dispersion with 4 mL of Tris-Gly buffer and 0.05 mL of 2-mercaptoethanol (ME). This was followed by the

addition of 12 % (w/v) trichloroacetic acid (TCA), and the mixture was then incubated for 1 h at 25°C. Subsequently, the sample was subjected to three cycles of centrifugation (4000 x g, 15 min) and resuspension in 12 % TCA to eliminate the ME. The resulting precipitate was resuspended in 10 mL Tris-Gly buffer. A 4 mL aliquot of this suspension was then treated with 0.04 mL of Ellman's reagent. Following a 20-min incubation period, the absorbance was measured at 412 nm using the Ultrospec 4300 Pro UV–Visible spectrophotometer (Amersham Biosciences, UK).

The content of free SH, total SH, and SS were calculated by equations (4), (5), and (6), respectively. In the equation, A is the absorbance at 412 nm, C is the sample concentration 7.50 mg/mL, D₁ is the dilution factor 5, D₂ is the dilution factor 10.

$$\text{Free SH } (\mu\text{mol/g}) = 73.53 \times A \times D_1 / C \quad (4)$$

$$\text{Total SH } (\mu\text{mol/g}) = 73.53 \times A \times D_2 / C \quad (5)$$

$$\text{SS } (\mu\text{mol/g}) = \text{Total SH} - \text{Free SH} / 2 \quad (6)$$

5.2.2.7 Intrinsic fluorescence

Intrinsic fluorescence measurements were conducted according to a modified protocol of Malomo and Aluko (2015). Protein stock solutions (10 mg/mL) were prepared in 0.1 M sodium phosphate buffers at pH 3.0, 5.0, 7.0, and 9.0. These mixtures were centrifuged, and the supernatants diluted to a final protein concentration of 0.002% (w/v). Fluorescence spectra were recorded using a Jasco FP 6300 spectrofluorometer (Jasco Inc., Tokyo, Japan) with an excitation wavelength of 280 nm and emission was scanned from 290 to 500 nm. To account for the background fluorescence, the spectrum of the corresponding buffer was subtracted from each sample spectrum.

5.2.2.8 Far UV and near UV circular dichroism

Far- and near-UV circular dichroism (CD) spectra were obtained as described by Osemwota et al. (2021), using a J-815 spectropolarimeter (Jasco Corporation, Tokyo, Japan) at a temperature of 25°C. Far-UV CD spectra were obtained for 2 mg/mL lupin protein fractions dispersed in buffer

at pH levels of 3, 5, 7, and 9, over a 190–240 nm wavelength range, using a quartz cuvette with a 0.5 mm path length. The near-UV spectra of lupin protein fractions were measured at wavelengths of 250–320 nm with a protein concentration of 4 mg/mL in a buffer (pH 3, 5, 7, and 9) using a 1 mm quartz cuvette. An average of five consecutive scans was obtained with simultaneous subtraction of the baseline (buffer). The secondary structure fractions were derived from the far-UV data using the SELCON3 algorithm (optimized for 190–240 nm) for secondary structure determination, as described by Miles et al. (2022) and available on the DichroWeb website (<http://dichroweb.cryst.bbk.ac.uk/html/home.shtml>). The reported β -sheet values represent combined parallel and anti-parallel β -sheet contributions, as the selected reference dataset did not resolve these structures separately.

5.2.2.9 Solubility

The solubility of lupin protein fractions was evaluated using the method described by Nwachukwu and Aluko (2018). For each sample, 50 mg of protein fraction was suspended in 5 mL of buffer solution with a pH value range of 3–9. The suspensions were thoroughly mixed by vortexing and allowed to hydrate for 1 h. After hydration, the mixtures were centrifuged at 5600× g for 30 min. The protein content of the resulting supernatants was quantified using the modified Lowry method, as described by Markwell et al. (1978). To determine the total protein content, 50 mg of a separate lupin protein fractions were completely solubilized in 5 mL of 1 M NaOH solution and the protein content was determined following the above procedure. Protein solubility (PS) was then calculated by comparing the protein content in the supernatant to the total protein content of the sample using equation (7).

$$PS (\%) = \frac{\text{protein content of the supernatant at a specific pH}}{\text{total protein content of the sample}} \times 100 \quad (7)$$

5.2.2.10 In vitro protein digestibility (IVPD)

IVPD of the protein fractions was evaluated using a modified version of the method described by Hsu et al. (1977). Protein fractions were dispersed in DDW and adjusted to pH 8.0 using 0.1 M NaOH while maintaining a temperature of 37°C under constant agitation. An enzymatic solution, kept in an ice bath, was prepared containing trypsin (1.6 mg/mL), chymotrypsin (3.1 mg/mL), and peptidase (1.3 mg/mL). A 3 mL portion of this enzyme mixture was introduced into 30 mL of each protein suspension. The pH of the resulting mixture was monitored and recorded at 30 s intervals for a total duration of 10 min using a pH meter. To ensure repeatability, the analysis was performed in duplicates. The percentage of protein digestibility for each sample was calculated (equation 8) using the regression equation proposed by Hsu et al. (1977).

$$\% \text{ Protein digestibility (Y)} = 210.46 - 18.10X_f \quad (8)$$

where X_f represents the final pH value of the sample after the 10-min digestion period.

5.2.2.11 Water and oil holding capacity

The water and oil-holding capacities of lupin protein fractions were evaluated using a modified version of the method described by Malomo and Aluko (2015). For oil holding capacity (OHC), protein fractions were prepared at 40 mg/mL concentrations in pre-weighed 15 mL centrifuge tubes. To each sample, 5 mL of canola oil was introduced. The resulting dispersion was vortexed for 1 min, followed by a 30-min resting period at ambient temperature. Subsequently, the mixture was centrifuged at 5600 x g for 30 min. The supernatant containing excess oil was then carefully drained for 15 min. The centrifuge tubes were weighed again to calculate the quantity of oil retained per gram of protein. The water holding capacity (WHC) was determined following a similar procedure, with the key difference being the replacement of canola oil with water. The process described for OHC was then replicated, allowing for assessment of the WHC.

5.2.2.12 Emulsion formation and stability

Oil-in-water emulsions were prepared using a modified version of the method described by Asen and Aluko (2023). Protein solutions were prepared at concentrations of 10, 15, and 20 mg/mL in different buffers: 0.1 M acetate buffer (pH 3), phosphate buffer (pH 5 and 7), and Tris-HCl buffer (pH 9). To create each emulsion, 1 mL of refined commercial canola oil was combined with 5 mL of protein solution. The mixture was then homogenized for 1 min at 20,000 rpm using a Polytron PT 10-35 homogenizer (Kinematica AG, Lucerne, Switzerland) fitted with a 12-mm generator. The mean surface-weighted oil droplet sizes ($d_{3,2}$) of the emulsions were measured using a Mastersizer 3000 (Malvern Panalytical Ltd., Malvern, UK). Milli-Q water was used as dispersant. The emulsion sample was gradually added to approximately 100 mL of water in the Hydro 3000 small-volume wet sample dispersion unit under constant shearing until the required obscuration level was reached. Triplicate measurements were performed and averaged. The droplet size measurement was repeated after the emulsion was left undisturbed at room temperature for 30 min to assess emulsion stability. Emulsion stability was calculated using equation (9):

$$\text{Emulsion stability (\%)} = \frac{\text{Mean oil droplet size at 0 min}}{\text{Mean oil droplet size at 30 min}} \times 100 \quad (9)$$

5.2.2.13 Foaming properties

The volume and stability of the foams generated by the lupin protein fractions were assessed following the procedure described by Nwachukwu and Aluko (2018). Protein solutions at concentrations of 10, 15, and 20 mg/mL were prepared in 5 mL of 0.1 M acetate (pH 3 and 5), phosphate (pH 7), and Tris-HCl (pH 9) buffers. The mixtures were then homogenized for 1 min using a Polytron PT 3100 (Kinematica AG, Lucerne, Switzerland) with a 12 mm shaft at 20,000 rpm. Foam height (cm) was recorded as the foaming capacity (FC), while foam stability (FS) was

determined as the volume after 30 min and expressed as a percentage of the initial foam height (cm).

5.2.2.14 Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics version 28 (Chicago, IL, USA). All measurements were performed in triplicate and reported as mean $\pm$ standard deviation. Data were first evaluated for normality using the Shapiro–Wilk test and for homogeneity of variance using Levene’s test. One-way analysis of variance (ANOVA) was performed, followed by Duncan’s multiple range test to identify statistically significant differences among means at $p < 0.05$. All graphs and data visualizations were generated using GraphPad Prism version 9 (San Diego, CA, USA).

5.3 RESULTS AND DISCUSSION

5.3.1 Protein extraction and fractionation

The protein and gross yields, which measure the proportion of total protein extracted in the fractions compared to the protein content in the original lupin flour and the total weight of the protein fraction in relation to the initial flour mass respectively, are shown in Table 5.1. White lupin globulin had the highest gross and protein yields, confirming that globulins are the predominant storage proteins in white lupins. This finding aligns with Duranti et al. (2008) and Shrestha et al. (2021) who reported that globulins are the predominant protein fractions in lupin seeds, accounting for over 80% of the total protein fractions. However, blue lupin exhibited unexpectedly high glutelin gross yield (37.5 g/100 g) and protein yield (14 g/100 g), which is unusual because the globulins have been reported to be the most predominant fractions in lupins. This anomaly may reflect cultivar-specific adaptations or differential protein body organization during seed development (Muranyi et al., 2016). It is also possible that incomplete globulin extraction, leading to protein redistribution into subsequent alkaline extractions may have

contributed to the high glutelin yield of the blue lupin. The substantially lower albumin yields in both species correspond with values reported in the literature, indicating that albumins typically represent 10-15% of the total seed proteins (Albe-Slabi et al., 2022). The prolamin content was too low and considered insignificant and has not been reported in this study. Protein content analysis supports the extraction efficiency, with white lupin globulin showing the highest purity (88.23%), while blue lupin albumin demonstrated the lowest (65.98%) protein content, which may limit its application in food formulations. A high protein content is important for ingredient functionality because contamination by fibers or minerals can interfere with the functional properties of proteins, such as gelation and emulsification (Vogelsang-O'Dwyer et al., 2020). Species variation significantly affects fraction distribution, with white lupin favoring conventional globulin accumulation, whereas blue lupin exhibits a more balanced globulin-glutelin distribution. These differences suggest that protein fraction selection should consider the species origin, as blue lupin glutelins may offer unique functional properties that are absent in traditional globulin-focused applications.

TABLE 5.1 Gross yield, protein recovery, and protein content of white and blue lupin globulin, albumin, and glutelin protein fractions

Sample name	Gross Yield (g/100g)	Protein yield (g/100g)	Protein content (% wet basis)
White Lupin Globulin (WLGlo)	16.71 ± 0.64 ^a	42.41 ± 1.63 ^a	88.23 ± 1.05 ^a
Blue Lupin Globulin (BLGlo)	13.27 ± 0.46 ^b	35.17 ± 1.22 ^c	80.47 ± 0.91 ^b
White Lupin Albumin (WLAib)	7.08 ± 0.56 ^c	16.55 ± 1.32 ^d	81.04 ± 1.16 ^b
Blue Lupin Albumin (BLAib)	2.77 ± 0.28 ^d	6.05 ± 0.62 ^e	65.98 ± 0.57 ^d
White Lupin Glutelin (WLGlu)	8.36 ± 0.37 ^c	17.79 ± 0.79 ^d	73.94 ± 0.36 ^c
Blue Lupin Glutelin (BLGlu)	14.00 ± 0.32 ^b	37.50 ± 0.87 ^b	81.39 ± 0.36 ^b

Values are represented as mean ± standard deviation. Values with different superscripts within the same column are significantly different ($p < 0.05$) as determined by ANOVA and Duncan's multiple range test.

5.3.2 Amino acid composition

The amino acid profile of the lupin protein fractions (Table 5.2) showed that the highest concentrations of amino acids were the non-essential amino acids glutamic acid (11–22 g/100 g) and aspartic acid (5–10 g/100 g), which are comparable to values reported for other legumes, such as faba (Mayer Labba et al., 2021), pea (Sá et al., 2024), and chickpea (Onder et al., 2023) proteins. These high levels are advantageous because glutamate and aspartate serve as the preferred energy sources for intestinal metabolism and support optimal gastrointestinal function (Deng et al., 2023). The abundance of these amino acids across lupin fractions indicates their potential to maintain optimal digestive health. Arginine emerged as the second most abundant amino acid across the fractions, consistent with typical legume protein profiles (Osemwota et al., 2021). The blue lupin glutelin had the highest arginine content, which is significantly higher than that of the albumin fractions. This conditionally essential amino acid generates nitric oxide, which promotes endothelial function, insulin secretion, and pancreatic beta cell protection (Martí & Reith, 2021; Wu et al., 2021). Arginine supplementation has positively affected type II diabetes mellitus by enhancing energy expenditure, improving glucose homeostasis, and reducing blood lipids and pressure (Forzano et al., 2023). Amino acid scoring against FAO/WHO adult requirements revealed methionine + cysteine and valine as the primary limiting factor across most fractions, consistent with typical legume protein nutrient limitations (Sá et al., 2024). Sulfur containing amino acids also serve as substrates for glutathione biosynthesis, providing antioxidant protection against oxidative damage, and supporting male fertility (Ahmed et al., 2023). While white lupin globulin was above the recommended score, with a score of 107 %, all other lupin protein fractions remained deficient (score 61–80 %). The white lupin globulin fractions with better methionine and cysteine contents may have a stronger nutritional appeal because elevated levels also confer enhanced antioxidant potential (Ahmed et al., 2023). Conversely, other essential amino acids, such

as phenylalanine + tyrosine, threonine, histidine, and lysine, exceeded the FAO/WHO requirements for most lupin protein fractions. Branched-chain amino acids showed species-specific variations in amino acid content. White lupin globulin had the highest leucine content and based on the amino acid scores for leucine and isoleucine, these two amino acids were only deficient in white lupin albumin. The amino acid scores for leucine and isoleucine for all fractions, except BLAlb, consistently exceeded 100, indicating adequate levels for muscle protein biosynthesis and metabolism. It is important to note that all the lupin protein fractions were deficient in valine based on the amino acid score. This is highly significant since valine deficiency is implicated in several physiological processes such as protein synthesis and muscle function, neurological effects, and impaired energy metabolism (Kim et al., 2022).

TABLE 5.2 Amino acid profile of white and blue lupin globulin, albumin, and glutelin protein fractions

	WLGLO		BLGLO		WLALB		BLALB		WLGLU		BLGLU	
Indispensable/conditionally Indispensable amino acid	Level (g/100g Protein)	AA Score % mg/g	Level (g/100g Protein)	AA Score % mg/g	Level (g/100g Protein)	AA Score % mg/g	Level (g/100g Protein)	AA Score % mg/g	Level (g/100g Protein)	AA Score % mg/g	Level (g/100g Protein)	AA Score % mg/g
Histidine	2.11 ± 0.04 ^b	132 ^γ	2.47 ± 0.00 ^{ab}	154 ^α	1.62 ± 0.02 ^c	101 ^ε	1.77 ± 0.01 ^d	111 ^δ	1.82 ± 0.03 ^c	113 ^δ	2.19 ± 0.02 ^b	137 ^β
Threonine	2.70 ± 0.01 ^b	108 ^γ	2.67 ± 0.02 ^b	107 ^γ	2.12 ± 0.01 ^c	85 ^δ	2.81 ± 0.10 ^{ab}	112 ^β	2.92 ± 0.01 ^a	117 ^α	2.97 ± 0.03 ^a	119 ^α
Lysine	3.61 ± 0.00 ^{cd}	75 ^ε	4.18 ± 0.11 ^b	87 ^β	3.34 ± 0.00 ^d	70 ^κ	4.83 ± 0.16 ^a	101 ^α	4.05 ± 0.06 ^b	84 ^γ	3.89 ± 0.06 ^{bc}	81 ^δ
Methionine + Cysteine	2.46 ± 0.02 ^a	107 ^α	1.85 ± 0.04 ^b	80 ^β	1.79 ± 0.02 ^b	78 ^γ	1.48 ± 0.01 ^c	64 ^δ	1.86 ± 0.02 ^b	81 ^β	1.41 ± 0.01 ^c	61 ^ε
Valine	3.11 ± 0.02 ^c	78 ^δ	3.29 ± 0.05 ^b	82 ^γ	2.50 ± 0.04 ^d	63 ^κ	2.98 ± 0.05 ^c	75 ^ε	3.40 ± 0.00 ^{ab}	85 ^β	3.48 ± 0.03 ^a	87 ^α
Isoleucine	4.04 ± 0.03 ^a	135 ^α	4.02 ± 0.06 ^a	134 ^α	3.48 ± 0.01 ^c	116 ^γ	2.50 ± 0.04 ^d	83 ^δ	3.50 ± 0.00 ^c	117 ^γ	3.65 ± 0.03 ^b	122 ^β
Leucine	7.66 ± 0.04 ^a	126 ^α	7.16 ± 0.12 ^b	117 ^β	6.26 ± 0.02 ^c	103 ^δ	3.99 ± 0.05 ^d	65 ^ε	6.45 ± 0.01 ^c	106 ^γ	6.45 ± 0.10 ^c	106 ^γ
Phenylalanine + Tyrosine	7.84 ± 0.04 ^a	191 ^α	7.63 ± 0.06 ^a	186 ^β	7.11 ± 0.01 ^a	173 ^γ	5.07 ± 0.02 ^c	124 ^ε	6.52 ± 0.02 ^b	159 ^δ	6.52 ± 0.05 ^b	159 ^δ
Tryptophan	0.61 ± 0.02 ^a	92 ^γ	0.57 ± 0.02 ^a	86 ^δ	0.29 ± 0.01 ^a	44 ^ε	0.81 ± 0.01 ^a	123 ^β	0.61 ± 0.00 ^a	92 ^γ	0.92 ± 0.00 ^a	139 ^α
Dispensable amino acids												
Serine	4.61 ± 0.07 ^a		4.51 ± 0.05 ^a		4.03 ± 0.00 ^b		2.51 ± 0.01 ^d		3.82 ± 0.00 ^c		3.84 ± 0.06 ^{bc}	
Arginine	8.54 ± 0.10 ^b		9.15 ± 0.11 ^a		7.09 ± 0.02 ^c		4.27 ± 0.19 ^c		6.19 ± 0.06 ^d		9.34 ± 0.24 ^a	
Glycine	2.69 ± 0.02 ^c		2.98 ± 0.02 ^b		2.21 ± 0.01 ^d		2.85 ± 0.08 ^b		2.67 ± 0.01 ^c		3.23 ± 0.03 ^a	
Aspartic acid	10.22 ± 0.06 ^a		9.84 ± 0.36 ^a		9.06 ± 0.05 ^b		5.61 ± 0.25 ^d		8.08 ± 0.00 ^c		8.83 ± 0.20 ^b	
Glutamic acid	22.82 ± 0.12 ^a		20.31 ± 0.60 ^b		19.8 ± 0.05 ^b		11.00 ± 0.51 ^d		14.97 ± 0.00 ^c		20.26 ± 0.64 ^b	
Proline	3.47 ± 0.02 ^{ab}		3.45 ± 0.05 ^b		2.66 ± 0.00 ^d		2.50 ± 0.06 ^c		2.84 ± 0.01 ^c		3.60 ± 0.02 ^a	
Alanine	2.25 ± 0.02 ^c		2.48 ± 0.05 ^b		1.89 ± 0.00 ^d		2.78 ± 0.09 ^a		2.59 ± 0.00 ^b		2.79 ± 0.02 ^a	

Values are represented as mean ± standard deviation. Values with different superscripts (letters and symbols) within the same row are significantly different ($p < 0.05$) as determined by ANOVA and Duncan's multiple range test.

TABLE 5.3 Surface hydrophobicity, thermal, functional, *in vitro* protein digestibility and sulfhydryl group and disulfide linkages of WLPI and BLPI

Parameters	WLGlo	BLGlo	WLA1b	BLA1b	WLGlu	BLGlu
<i>Surface hydrophobicity (S_o)</i>						
	609.05 $\pm$ 8.00 ^c	641.59 $\pm$ 1.34 ^d	691.12 $\pm$ 13.49 ^c	366.54 $\pm$ 11.72 ^f	1232.90 $\pm$ 27.20 ^b	3924.10 $\pm$ 22.70 ^a
<i>Thermal properties</i>						
T_o Onset ($^{\circ}\text{C}$)	90.50 $\pm$ 0.59 ^b	84.62 $\pm$ 0.09 ^d	97.03 $\pm$ 0.63 ^a	71.58 $\pm$ 0.58 ^c	87.20 $\pm$ 1.03 ^c	88.55 $\pm$ 0.53 ^c
Maximum T_d ($^{\circ}\text{C}$)	100.30 $\pm$ 0.57 ^b	98.59 $\pm$ 0.21 ^c	103.84 $\pm$ 0.57 ^a	74.01 $\pm$ 0.21 ^c	92.35 $\pm$ 0.35 ^d	92.96 $\pm$ 0.09 ^d
ΔH (J/g)	4.95 $\pm$ 0.32 ^b	6.37 $\pm$ 0.25 ^a	5.24 $\pm$ 0.92 ^b	0.61 $\pm$ 0.51 ^c	0.41 $\pm$ 0.04 ^c	0.16 $\pm$ 0.03 ^c
<i>Functional properties</i>						
OHC (g/g)	3.02 $\pm$ 0.06 ^c	3.63 $\pm$ 0.11 ^c	6.44 $\pm$ 0.06 ^b	11.61 $\pm$ 0.34 ^a	10.70 $\pm$ 0.08 ^a	10.55 $\pm$ 0.64 ^a
WHC (g/g)	1.14 $\pm$ 0.06 ^b	1.56 $\pm$ 0.07 ^b	0.36 $\pm$ 0.03 ^b	0.79 $\pm$ 0.10 ^b	3.10 $\pm$ 0.04 ^a	1.26 $\pm$ 0.15 ^b
<i>In vitro protein digestibility</i>						
IVPD (%)	73.08 $\pm$ 0.18 ^d	84.65 $\pm$ 0.95 ^b	72.71 $\pm$ 0.18 ^d	72.08 $\pm$ 0.45 ^d	80.77 $\pm$ 0.09 ^c	92.44 $\pm$ 2.17 ^a
<i>Sulfhydryl group (SH) and disulfide linkages (SS)</i>						
Free SH ($\mu\text{mol/g}$)	48.44 $\pm$ 0.18 ^a	44.39 $\pm$ 0.19 ^b	27.87 $\pm$ 0.02 ^c	17.33 $\pm$ 0.17 ^d	10.53 $\pm$ 0.04 ^c	11.38 $\pm$ 0.09 ^c
Total SH ($\mu\text{mol/g}$)	85.58 $\pm$ 4.42 ^c	105.91 $\pm$ 4.78 ^b	118.08 $\pm$ 0.04 ^a	66.30 $\pm$ 4.89 ^d	66.99 $\pm$ 4.87 ^d	53.56 $\pm$ 6.09 ^c
Disulfide bond ($\mu\text{mol/g}$)	18.57 $\pm$ 2.21 ^c	30.76 $\pm$ 2.39 ^b	45.11 $\pm$ 0.01 ^a	24.48 $\pm$ 2.51 ^c	28.22 $\pm$ 2.42 ^b	21.08 $\pm$ 3.09 ^d

Values are represented as Mean $\pm$ standard deviation. Values with different superscripts within the same row differ significantly ($P < 0.05$). T_o : onset denaturation temperature, T_d : maximum denaturation temperature; ΔH : enthalpy of denaturation; OHC: oil holding capacity; WHC: water holding capacity; IVPD: *In-vitro* protein digestibility. WLGlo-white lupin globulin, BLGlo-blue lupin globulin, WLA1b-white lupin albumin, BLA1b-blue lupin albumin, WLGlu-white lupin glutelin, BLGlu-blue lupin glutelin.

5.3.3 Gel electrophoresis

SDS-PAGE is crucial in determining the molecular composition, structural characteristics, and polypeptide distribution patterns of lupin protein fractions. This analysis is essential for understanding the diversity of polypeptides and identifying the major protein bands in each fraction. Under reducing conditions (Fig. 5.1A), the major protein bands appeared in the 15-100 kDa range, consistent with the subunit composition of lupin conglutins as previously reported (Foley et al., 2015). For the globulin fractions (lanes f and g), the predominant bands at approximately 50-60 kDa and 20-25 kDa correspond to the acidic (α) and basic (β) subunits of α -conglutin (legumin-like, 11S globulin), while bands around 15-20 kDa, 25-45 kDa, and 53-64 kDa represent the low, intermediate, and high relative molecular weight subunit 7S (vicilin-like), respectively. Albumin fractions (lanes d, e) display distinctly different profiles with more prominent low molecular weight bands (15-25 kDa), consistent with the presence of γ - and δ -conglutin, which supports the enhanced solubility characteristics observed in the albumin fractions. The presence of a high molecular weight $\sim$ 100 kDa (lipoxygenase) band may also limit the application of this fraction in food formulations that are not thermally treated, as aroma compounds produced during lipoxygenase-catalyzed reaction are known to impart undesirable flavor characteristics (Shi et al., 2020). Interestingly, this lipoxygenase band was found only in the albumin fraction, suggesting it is tightly bound to this fraction. The glutelin fractions displayed significantly different band patterns under reducing conditions. For BLGlu, prominent bands were found at 15-20 kDa, 25-45 kDa, and 53-64 kDa, indicating the presence of 7S vicilin subunits, whereas for WLGLu, the predominant band could be found at 25 kDa. Under nonreducing conditions (Fig. 5.1B), several bands appeared at higher molecular weights, indicating disulfide-linked protein complexes that dissociated upon reduction. This is also evident in the glutelin fractions, where WLGLu displayed only a predominant 50 kDa band, and when compared with the

result from reducing SDS-PAGE, the predominant band for WLGl_u is 25 kDa, suggesting the cleavage of disulfide bonds anchoring the subunit together. Although α -conglutin is known to form native hexameric assemblies (300–400 kDa), these structures are typically not preserved during SDS-PAGE. The hexamer is stabilized mainly by non-covalent interactions that are disrupted by SDS, resulting in dissociation into the constituent acidic (~40 kDa) and basic (~20 kDa) subunits, or their disulfide-linked pairs (~60 kDa). Consequently, the intact hexamer was not visible in the gel, and only lower-molecular-weight subunits were observed, which is consistent with the expected electrophoretic behavior of 11S globulins (Duranti et al., 2008). In addition, the overlap in molecular weight ranges may indicate shared protein components across fractions, particularly between albumin and globulins, suggesting incomplete separation during fractionation.

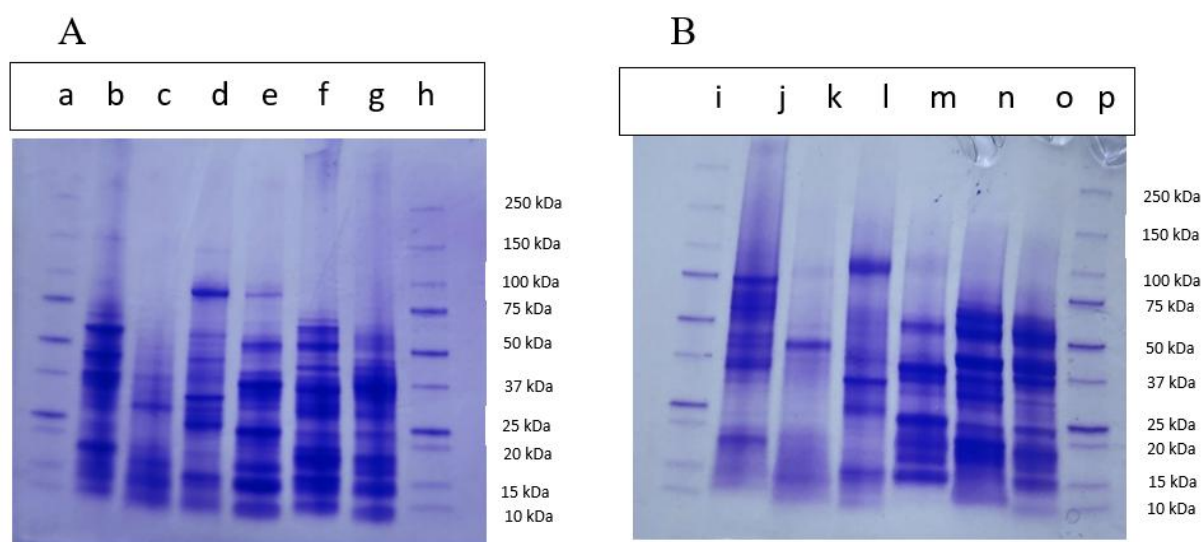


FIGURE 5.1 SDS PAGE of white and blue lupin albumin, globulin, and glutelin fractions. **A.** Under reducing conditions. **a:** Standard protein markers (kDa); **b:** reduced BLGl_u; **c:** reduced WLGl_u; **d:** reduced BLAlb; **e:** reduced WLAlb; **f:** reduced BLGl_o; **g:** reduced WLGl_o; **h:** Standard protein markers (kDa). **B** Under non-reducing conditions. **i:** Standard protein markers (kDa); **j:** non-reduced BLGl_u; **k:** non-reduced WLGl_u; **l:** non-reduced BLAlb; **m:** non-reduced WLAlb; **n:** non-reduced BLGl_o; **o:** non-reduced WLGl_o; **p:** Standard protein markers (kDa).

5.3.4 Surface hydrophobicity

Surface hydrophobicity (S_o) represents a critical structural parameter that affects protein functionality in food systems by quantifying the exposure of hydrophobic amino acid residues on protein surfaces. Blue lupin glutelin demonstrated high surface hydrophobicity, which is significantly higher than all other fractions and surpassed the values reported for fava, lentil, and chickpea glutelin (Oluwajuyitan & Aluko, 2025; Osemwota et al., 2022; Perović et al., 2025). The high surface hydrophobicity (Table 5.3) of BLGlu may result from extensive protein unfolding during alkaline extraction, thereby exposing buried hydrophobic residues that are usually sequestered in the protein core. The alkaline extraction conditions required for BLGlu isolation may disrupt the tertiary structure, creating an expanded conformation with enhanced hydrophobic surface area (Perović et al., 2025). Globulin fractions showed moderate surface hydrophobicity (609 - 641). This aligns with their globular, highly folded structure compared to the more extended conformations of the albumin and glutelin fractions. The compact globulin architecture typically maintains hydrophobic amino acids in the protein interior, with polar residues dominating the surface for aqueous stability. Comparing the blue lupin proteins, the albumin exhibited the lowest surface hydrophobicity, which correlates with its low content of hydrophobic amino acids, as indicated by the amino acid profiles (Table 5.2). Although BLAlb contained a low content of hydrophobic amino acids, the results also suggested that the hydrophobic residues could be buried within the protein core of denatured albumin structures (Perović et al., 2025). The presence of hydrophilic carbohydrate moieties in albumin may dominate the outer surface, effectively shielding hydrophobic groups from the aqueous environment and reducing the overall surface hydrophobicity measurements, which may explain the high solubility characteristics observed in albumin fractions (Oluwajuyitan & Aluko, 2025). The glutelin > globulin > albumin S_o trend obtained in this study is similar to previously reported studies for pea and chickpea (Lam et al.,

2018; Perović et al., 2025). The effect of lupin species was observed in the surface hydrophobicity patterns of the protein fractions. For the glutelin fractions, BLGlu exhibited a high H_0 value—over threefold higher than WLGl—indicating a greater degree of unfolding and hydrophobic residue exposure during alkaline extraction. In contrast, the albumin fractions demonstrated the opposite trend: BLAlb showed the lowest H_0 among all fractions and was markedly lower than WLAlb, suggesting that blue lupin albumin possesses more shielded hydrophobic regions or a more compact conformation. The globulin fractions also reflected species-related differences, with BLGlo showing moderately higher H_0 than WLGlo. Collectively, these results demonstrate that lupin species significantly influence the structural exposure of hydrophobic residues across protein classes, leading to distinct functional behaviors.

5.3.5 Sulfhydryl group content

Sulfhydryl groups (free SH) and disulfide linkages (SS) are critical structural components that govern protein tertiary and quaternary structures, directly influencing protein stability, functionality, and processing behavior. Free sulfhydryl groups provide reactive sites for protein-protein interactions, oxidation reactions, and cross-linking during thermal processing, whereas disulfide bonds contribute to overall protein structural integrity and thermal stability (Wang et al., 2009). The balance between free SH groups and disulfide linkages determines protein susceptibility to oxidative modifications, heat-induced gelation, and changes in functional properties during processing and storage. The results of the sulfhydryl groups and disulfide linkages are presented in Table 5.3, with white lupin globulin exhibiting the highest free sulfhydryl content (48.44 $\mu\text{mol/g}$), which is significantly higher than that of the albumin and glutelin fractions. The glutelin fractions had the lowest free SH content, and there was no significant difference in free SH content between the blue and white lupin glutelin fractions. The low free SH

content in the glutelin may be an indication of extensive oxidation or cross-linking during the alkaline extraction processes, which is consistent with reports by Nasrabadi et al. (2021) who demonstrated that harsh extraction conditions promote sulfhydryl oxidation and disulfide bond formation in legume proteins. The substantial difference between total and free SH content indicates extensive disulfide cross-linking within protein structures, particularly evident in albumin fractions, where disulfide bonds (45.11 $\mu\text{mol/g}$) represent approximately 38% of total sulfur groups. Glutelin fractions showed reduced total SH content (53.56-66.99 $\mu\text{mol/g}$) and moderate disulfide bonding (21.08-28.22 $\mu\text{mol/g}$), suggesting that alkaline extraction conditions promote sulfur group modifications and potential disulfide mediated aggregation (Nasrabadi et al., 2021). These sulfur group characteristics have significant implications for food processing and functionality. High free SH content in globulin fractions may increase susceptibility to oxidative modifications during processing and storage, requiring careful handling and antioxidant protection (Colovic et al., 2018). The significant differences in sulfhydryl and disulfide contents between white and blue lupins arise from species-specific variations in their protein subunit composition and structural organization. These intrinsic differences influence both the number and accessibility of cysteine residues. This is supported by electrophoresis profiles, in which each species shows distinct band patterns and intensities that reflect differences in molecular weight distribution, degree of subunit polymerization, and susceptibility to disulfide-mediated aggregation. Proteins with more accessible cysteine-containing subunits appear as clearer, well-defined bands under reducing conditions, whereas proteins with more extensive disulfide cross-linking present as higher molecular weight aggregates or smeared bands under non-reducing conditions (Duranti et al., 2008).

5.3.6 Differential scanning calorimetry

The thermal properties of proteins, including denaturation temperature (T_d), onset temperature (T_o), and enthalpy of denaturation (ΔH), are essential parameters for understanding protein structural stability and processing behavior. These parameters directly influence food processing conditions, shelf stability, and functional property retention during thermal treatments such as pasteurization, cooking, and sterilization (Devkota et al., 2023). Understanding thermal transitions enables the optimization of processing parameters to maintain protein functionality while ensuring food safety and serves as an indicator of protein structural integrity and resistance to heat-induced modifications. The DSC results (Table 5.3 and Figures S2-S13) showed that WLAlb had the highest denaturation temperature (103°C), which is significantly higher than those of the other fractions. The high denaturation temperature of WLAlb correlates with its high disulfide-bond content, as higher levels increase protein stability and resistance to unfolding. The reverse was observed for BLAlb, where the denaturation temperature was significantly lower and aligned with findings by Oluwajuyitan and Aluko (2025), who reported similar low thermal stability in albumin-rich protein fractions due to low disulfide cross-linking and a flexible molecular structure. Although the glutelin fractions did not exhibit the highest denaturation temperatures, their T_d values remained similar ($92\text{--}93^\circ\text{C}$) for both lupin species. This indicates that the glutelin proteins retained similar conformation, although they are less thermally stable than WLGl and WLAlb. The comparable T_d values of WLGl and BLGl suggest that, despite extensive unfolding during alkaline extraction, the glutelin proteins retain thermally more resistant structures than the BLAlb through intermolecular associations formed during processing (Devkota et al., 2024). The enthalpy values revealed significant structural differences, with white lupin albumin showing the highest ΔH (5.24 J/g), indicating substantial energy requirements for protein unfolding and supporting its superior thermal stability. The relatively low enthalpy values in glutelin fractions

(0.16-0.41 J/g) indicate minimal energy requirements for further structural transitions, suggesting that extensive unfolding during extraction has already disrupted most ordered protein structures. The thermal stability patterns, together with the favorable solubility characteristics of WLA1b, are advantageous for high-temperature processing applications, where protein functionality must be maintained while preserving nutritional qualities under severe thermal stress.

5.3.7 Intrinsic fluorescence

Intrinsic fluorescence spectroscopy provides insight into the conformational environment of aromatic amino acid residues, particularly tryptophan and tyrosine, and is a sensitive probe for tertiary structure changes in proteins (Cheng et al., 2020). Across the range of pH values tested, marked differences in fluorescence intensity were observed between the white and blue lupin protein fractions (Fig. 5.2), reflecting pH-dependent structural rearrangements and differential exposure of fluorophores. At pH 3, all fractions exhibited red-shifted emission and relatively high fluorescence intensity, with BLGlu showing the strongest signal. This suggests a significant exposure of tryptophan residues to polar environments, likely due to partial unfolding under acidic conditions (Kyrychenko & Ladokhin, 2023). The albumin fractions (WLA1b and BLA1b) displayed lower fluorescence, consistent with a less compact or more solvent-exposed tertiary structures at pH 3. At pH 5, which is close to the isoelectric point of most legume proteins, a general decrease in fluorescence intensity was observed in the globulin and glutelin fractions. This could be attributed to protein aggregation, which can lead to burying of tryptophan residues and enhanced quenching effects (Kyrychenko & Ladokhin, 2023). Notably, BLA1b exhibited the highest fluorescence at this pH, suggesting a species- or fraction-specific resistance to aggregation-induced quenching. Moderate recovery of fluorescence was observed across all fractions at pH 7, with BLGlo and WLGlo displaying peak intensities. This may reflect increased protein solubility

and tertiary rearrangement into more compact conformations, allowing stronger synergistic interactions between the fluorophores. This behavior aligns with findings in legume proteins, such as soy and pea, where neutral pH promotes solubility and an increase in flexible tertiary structure (Liang & Tang, 2013). The fluorescence intensity further increased at pH 9, particularly in BLGlo and BLAlb, reflecting increased solubility that pushed the tryptophan residues away from the hydrophilic environment, which avoids fluorescence quenching. This behavior is consistent with previous reports, which show enhanced fluorescence at alkaline pH, indicative of tertiary structure rearrangement (Li et al., 2021). The high fluorescence intensity of BLGlo suggests that blue lupin globulin is particularly responsive to pH-induced conformational changes. Overall, the blue lupin protein fractions consistently exhibited higher fluorescence intensities across all pH conditions, indicating conformations with less exposure of the aromatic groups to the hydrophilic environment when compared to the white lupin protein fractions.

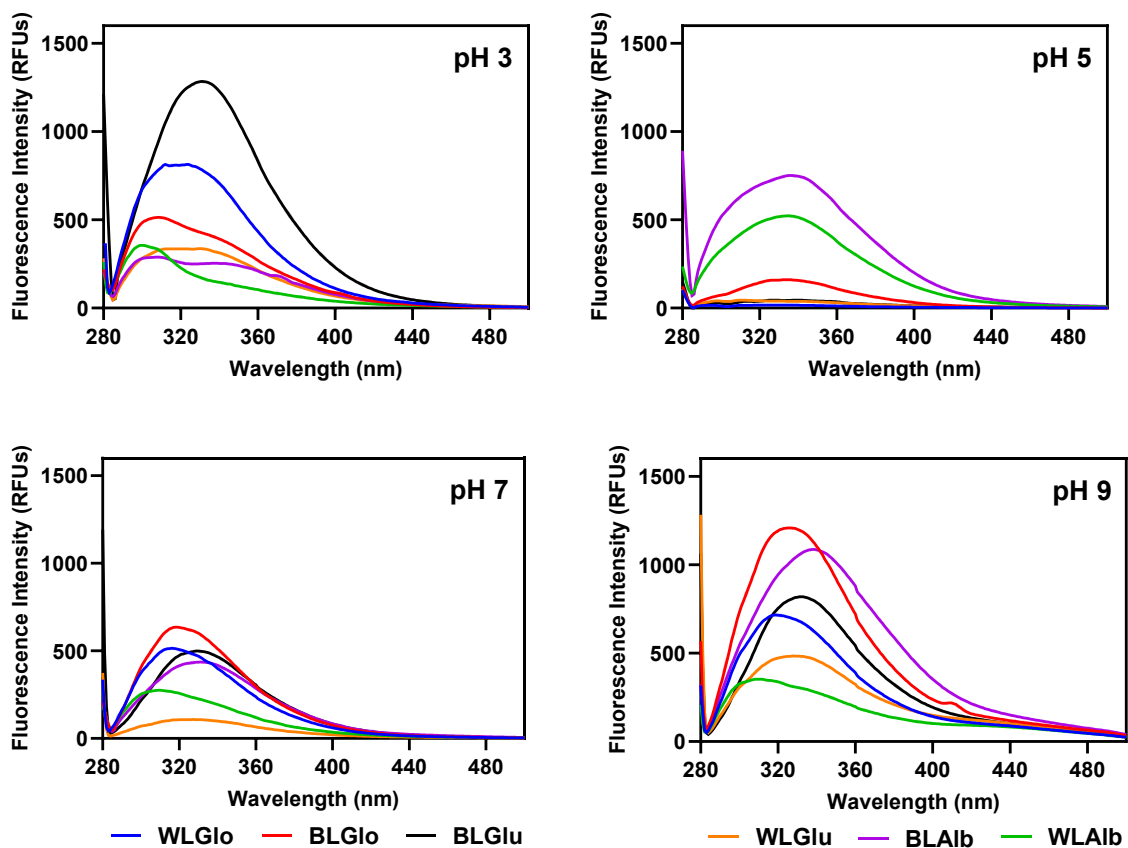


FIGURE 5.2 Intrinsic fluorescence intensity as relative fluorescence units (RFUs) at different pH values of the white and blue lupin protein fractions. WLGlO-white lupin globulin, BLGlO-blue lupin globulin, WLAlb-white lupin albumin, BLAlb-blue lupin albumin, WLGlU-white lupin glutelin, BLGlU-blue lupin glutelin.

5.3.8 Far-UV and near-UV circular dichroism (CD)

Near-UV CD spectroscopy offers insights into the protein's tertiary structure by examining the asymmetric environment of aromatic amino acid residues (tyrosine, tryptophan, and phenylalanine), revealing pH-induced conformational changes that impact protein function and stability (Hoffmann et al., 2016). pH greatly influences plant protein structure by altering the protonation of ionizable amino acids, with approximately one-third of these amino acids having

pH-sensitive side chains (Arakawa et al., 2021). The near-UV spectra (Figure 5.3) showed that at pH 3, the most notable feature was the strong negative ellipticity minimum around 250 nm in the glutelin fractions, indicating considerable structural disturbance under acidic conditions. This intense negative signal suggests that aromatic amino acids, especially phenylalanine, experience a shift into the protein interior, which reduces interactions with the hydrophilic environment (Li et al., 2011). The drop in ellipticity that occurs between ~250 nm to 270 nm indicates partial protein unfolding, with aromatic residues becoming more exposed to the aqueous environment, suggesting that glutelin fractions were particularly sensitive to acidic conditions. The spectral values at pH 5 revealed reduced ellipticity values approaching zero for the globulins and glutelin fractions, indicating a transition toward more open protein conformations that have extensive contacts with the hydrophilic environment. This pH represents a critical transition zone where the reduced net charge minimizes electrostatic repulsion, allowing proteins to adopt more aggregated and denatured structures. The diminished CD signals suggest that aromatic residues experience reduced asymmetry in the microenvironment, indicating partial unfolding and aggregation, which is a sign of tertiary structure disruption (Arakawa et al., 2021). At pH 7, all lupin protein fractions exhibited distinct near-UV CD spectra, indicating a more defined tertiary structure compared to the spectra observed at pH 5. The peak intensities between 270 and 290 nm indicate asymmetrical environments for tyrosine and tryptophan residues, consistent with native-like conformations (Kelly & Price, 1997; Schmid, 1989). Among all fractions, BLGlu and WLGlU displayed the highest ellipticity values, signifying a more compact tertiary structure with reduced exposure to the hydrophilic environment. In contrast, the BLAlb and WLAlb fractions displayed lower ellipticity values, suggesting less-defined tertiary structures. The relatively flat spectra of BLAlb and WLAlb also suggest possible partial denaturation at pH 7, which may indicate disruption of

tertiary structure, probably due to high solubility in the aqueous environment. Interestingly, WLGu and BLGu also showed moderate increases in ellipticity at pH 9 compared to pH 7, suggesting that the fractions, which are typically less flexible due to extensive disulfide bonding, might have attained conformations that moved the aromatic residues farther away from the aqueous environment under the strong alkaline condition. The results obtained in this study are similar to previous research reports that also showed the effects of different pH levels and processing methods on protein structure (Aderinola et al., 2020; Devkota et al., 2023).

The secondary structure composition of the white and blue lupin protein fractions (Table 5.4) varied significantly across pH conditions, reflecting their structural diversity and conformational stability. At pH 3, most of the protein fractions showed a higher quantity of unordered structures ($\geq 56\%$) with relatively lower β -sheet and α -helix contents. Notably, BLGu and BLAlb exhibited the highest proportions of unordered structures (70.15% and 67%, respectively), indicating partially denatured or flexible conformations under acidic conditions. The observed secondary structure transitions likely resulted from pH-induced alterations in the protein charge distribution, affecting both electrostatic interactions and hydrogen bonding patterns within the protein matrix (Albe-Slabi et al., 2022). Upon shifting to pH 5, a substantial increase in the β -sheet content was observed in all the globulin and glutelin fractions. This elevation may be attributed to enhanced protein-protein

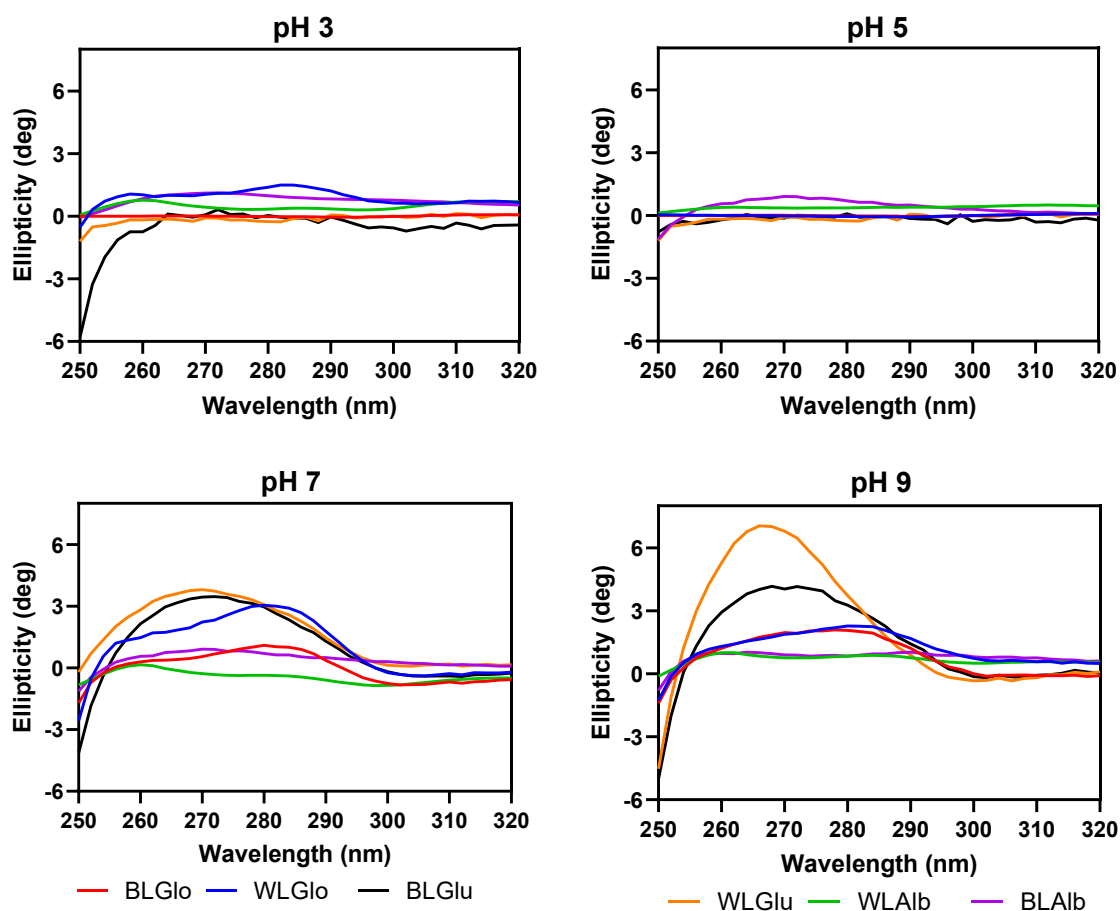


FIGURE 5.3 Near UV circular dichroism spectra at different pH values of the white and blue lupin protein fractions. WLGlo-white lupin globulin, BLGlo-blue lupin globulin, WLA1b-white lupin albumin, BLA1b-blue lupin albumin, WLGlu-white lupin glutelin, BLGlu-blue lupin glutelin.

TABLE 5.4 Secondary structure of white and blue lupin albumin, globulin, and glutelin at different pH values.

WLGlo				
pH	3	5	7	9
α -helix	21.00 ± 0.20^a	3.50 ± 1.00^d	18.60 ± 0.20^b	7.10 ± 2.80^c
β -sheet	5.75 ± 2.80^c	37.90 ± 1.60^a	2.95 ± 0.70^d	15.65 ± 5.40^b
Turns	11.95 ± 1.70^c	19.00 ± 0.20^a	9.35 ± 2.00^d	14.35 ± 1.60^b
Unordered	61.30 ± 4.80^b	39.65 ± 0.70^c	69.15 ± 2.50^a	62.90 ± 1.00^b
BLGlo				
pH	3	5	7	9
α -helix	16.55 ± 0.10^b	4.65 ± 1.75^d	18.85 ± 0.10^a	6.50 ± 3.20^c
β -sheet	3.55 ± 0.75^c	34.25 ± 2.30^a	2.90 ± 0.60^c	15.90 ± 4.40^b
Turns	9.75 ± 2.00^c	18.05 ± 0.10^a	9.60 ± 1.60^c	13.85 ± 2.30^b
Unordered	70.15 ± 2.60^a	43.10 ± 0.60^c	68.70 ± 2.20^a	63.65 ± 0.90^b
WLAib				
pH	3	5	7	9
α -helix	18.45 ± 0.10^b	9.15 ± 4.30^c	23.15 ± 0.10^a	18.75 ± 0.40^b
β -sheet	7.05 ± 1.60^a	8.65 ± 4.50^a	4.75 ± 2.30^b	2.55 ± 0.70^c
Turns	17.80 ± 1.10^a	14.70 ± 0.60^b	10.25 ± 1.70^c	8.65 ± 2.30^d
Unordered	56.75 ± 0.60^d	67.60 ± 8.30^b	61.85 ± 4.10^c	70.10 ± 2.70^a
BLAib				
pH	3	5	7	9
α -helix	9.50 ± 4.80^c	18.35 ± 0.15^a	18.00 ± 0.10^a	16.70 ± 0.20^b
β -sheet	9.55 ± 4.80^a	3.05 ± 0.90^b	3.35 ± 0.60^b	3.35 ± 0.70^b
Turns	14.00 ± 0.40^a	10.35 ± 2.50^b	10.25 ± 1.70^b	9.60 ± 2.20^b
Unordered	67.00 ± 9.20^b	68.30 ± 3.30^b	68.40 ± 2.30^b	70.30 ± 2.70^a
WLGlu				
pH	3	5	7	9
α -helix	9.40 ± 3.90^b	5.20 ± 2.60^c	5.95 ± 2.30^c	15.40 ± 0.20^a
β -sheet	10.15 ± 4.20^c	29.85 ± 1.60^a	24.00 ± 0.30^b	8.50 ± 1.90^d
Turns	16.25 ± 1.50^a	17.60 ± 0.40^a	14.95 ± 1.60^b	11.15 ± 2.40^c
Unordered	64.15 ± 6.60^a	47.30 ± 1.30^c	55.10 ± 4.40^b	64.95 ± 4.20^a
BLGlu				
pH	3	5	7	9
α -helix	17.00 ± 0.20^a	4.20 ± 1.30^c	8.80 ± 0.40^b	17.15 ± 0.10^a
β -sheet	7.05 ± 1.60^c	36.00 ± 2.20^a	11.40 ± 0.40^b	2.90 ± 0.60^d
Turns	11.35 ± 2.20^c	18.25 ± 0.20^b	20.50 ± 0.60^a	8.70 ± 2.00^d
Unordered	64.70 ± 3.60^b	41.60 ± 1.00^d	59.30 ± 2.00^c	71.20 ± 2.50^a

Values are represented as mean $\pm$ standard deviation. Values with different superscripts within the same column are significantly different ($p < 0.05$) as determined by ANOVA and Duncan's multiple range test.

interactions near their isoelectric points, which often promote β -sheet formation. Concomitantly, the α -helix and unordered content decreased, particularly in BLGlo and WLGlu, suggesting a loss of native conformation and partial rearrangement into β -rich structures. At pH 7, the globulin fractions (WLGlo and BLGlo) and albumins (WLA1b and BLA1b) exhibited a marked increase in α -helix content (up to 23.15% in WLA1b), while unordered structures remained nearly unchanged. This suggests a more native-like conformation at neutral pH, likely due to increased solubility and electrostatic repulsion that prevent extensive protein-protein interactions. The transition to pH 9 generally resulted in a decrease in β -sheet content and slight increases in α -helical structures, especially in BLGlu and WLGlu, which may reflect partial reorganization or unfolding under alkaline conditions. The WLA1b generally maintained relatively high α -helix content at pH 3, 7, and 9. A notable exception was observed at pH 5, where WLA1b showed a substantial reduction in α -helical content (9.15%), approximately a 50% decrease relative to pH 3. This marked structural disruption at pH 5 coincides with the protein's isoelectric point region, where reduced electrostatic repulsion and minimal net charge promote protein aggregation and partial unfolding. The concurrent increase in unordered structure at pH 5 (67.60%) compared to other pH values supports this interpretation. Beyond the isoelectric region (pH 7 and 9), WLA1b recovered its α -helical structure, suggesting that electrostatic effects primarily drive this pH-induced conformational change. This stability is characteristic of albumin proteins, which typically exhibit better solubility across varying pH conditions than other protein fractions (Ye et al., 2024). In contrast, the glutelin and globulin fractions exhibited higher β -sheet content at pH 5 and unordered structures under acidic conditions, as well as a low content of α -helices at pH 7, reflecting their pH-sensitive conformational behavior. This trend is consistent with observations in other legume proteins, such as those from lentil (Osemwota et al., 2022) and chickpea (Perović et al., 2025), where glutelin

fractions typically contain higher proportions of unordered conformations due to their extraction under harsh alkaline conditions.

5.3.9 Solubility

Protein solubility is essential for understanding protein functionality because it reveals the relationship between protein structure, interaction with the immediate environment, and functional behavior in food systems. Solubility profiles directly influence emulsification properties, foaming capacity, gelation properties, and industrial processing applications. The results in Figure 5.4 show the characteristic U-shaped solubility curves typical of legume proteins, with minimum solubility at pH 5.0 for globulins and glutelin, representing the isoelectric point where the net protein charge approaches zero. This behavior aligns with Ye et al. (2024), who reported similar solubility patterns for chickpea protein fraction between pH 4.5-5.5. The globulin fractions from both lupin species displayed comparable solubility patterns at acidic pH (pH 3), with BLGlo showing slightly higher solubility (86%) than WLGLo (78%). However, under acidic conditions, the BLGlu displayed a higher solubility (63%) than WLGLu (40%), indicating a varietal effect. Under neutral and alkaline conditions, the globulin fractions maintained significantly higher solubility (83-98%) than the glutelin fractions (48-81%), even though glutelin extraction was performed under alkaline conditions. This superior solubility can be attributed to several factors. Firstly, amino acid composition plays a key role. Globulin fractions contained higher levels of charged amino acids, particularly glutamic acid (WLGLO: 22.82 vs WLGLU: 14.97 g/100g protein) and aspartic acid (WLGLO: 10.22 vs WLGLU: 8.08 g/100g protein) (Table 5.2). At neutral and alkaline pH, these acidic residues are negatively charged, thereby enhancing electrostatic repulsions between protein molecules and promoting interactions with water. Additionally, globulins possess a more compact structure with lower aggregation tendencies compared to glutelins. The glutelin fractions may also

contain residual insoluble materials or have undergone partial denaturation during extraction, further reducing their solubility (Ye et al., 2024). For BLAlb, the solubility continued to increase with increasing pH, whereas for WLAlb, aside from a drop at pH 4-5, the solubility at the acidic and alkaline scales was high. Species-specific differences emerged prominently between the white and blue lupin fractions. White lupin albumin achieved maximum solubility (84-100% at pH 6-8), which significantly exceeded that of blue lupin albumin (77-93%). The superior performance of white lupin albumin suggests an enhanced surface charge distribution and reduced aggregation tendency under neutral-alkaline conditions. The enhanced solubility of albumin can be attributed to its reduced hydrophobic and aromatic amino acid content coupled with significantly elevated concentrations of negatively charged amino acids. Additionally, albumin's predominant composition of smaller molecular weight polypeptides promotes greater conformational mobility and aqueous dispersibility than the larger molecular weight structures characteristic of globulin fractions (Duranti et al., 2008). The solubility profile of the lupin protein fractions was similar to that previously reported for rice bran and flaxseed seed proteins (Nwachukwu & Aluko, 2018; Wang et al., 2015).

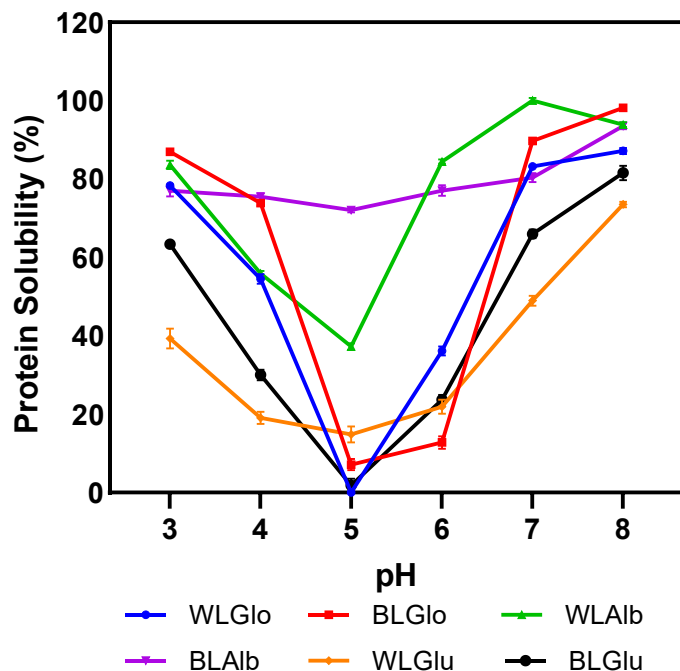


FIGURE 5.4 Percentage solubility profile of white and blue lupin protein fractions as a function of pH. WLGlo-white lupin globulin, BLGlo-blue lupin globulin, WLAib-white lupin albumin, BLAib-blue lupin albumin, WLGlu-white lupin glutelin, BLGlu-blue lupin glutelin.

5.3.10 In vitro protein digestibility (IVPD)

IVPD assesses protein nutritional quality and bioavailability by simulating gastrointestinal tract digestion to predict how effectively proteins can be broken down into absorbable amino acids and peptides. This measurement is essential for food product development as it directly influences protein utilization efficiency, nutritional value, and consumer health benefits. IVPD assessment enables rapid screening of protein quality without expensive animal studies, providing a framework for structure-digestibility relationships that guide processing optimization and formulation strategies. The IVPD results (Table 5.3) revealed significant variations in digestibility across lupin protein fractions, with blue lupin glutelin having the highest IVPD (92%), significantly exceeding those of the other fractions. The high digestibility of the BLGlu fractions correlates directly with their extensive structural modification during alkaline extraction and low

disulfide bond content. Protein unfolding exposes peptide bonds normally buried within compact protein structures, enhancing accessibility for pepsin and pancreatin enzymes during simulated digestion. This relationship between processing-induced structural changes and digestibility supports the findings of Chen et al. (2023), who demonstrated that thermal and chemical treatments improved legume protein digestibility through conformational modifications. There was no significant difference between the IVPD of the albumin and WLGlo fractions. The moderate digestibility in globulin fractions (72-84%) compares favorably with values reported for pea, fava, and lentil protein isolates (Carlin et al., 2025), positioning lupin proteins competitively within the plant protein market. The inverse relationship between compact protein structure and digestibility is evident when comparing albumin and globulin fractions. Although albumin has a lower molecular weight composition, which should theoretically enhance enzymatic access, the moderate digestibility values (72%) suggest that structural factors beyond molecular size influence digestibility, such as the presence of antinutritional factors like protease inhibitors, tannins, and phytates. The superior IVPD performance of lupin proteins compared to other legumes (Carlin et al., 2025), particularly in glutelin fractions, indicates significant potential for high-quality protein ingredient development. These digestibility characteristics and excellent amino acid profiles make lupin proteins valuable alternatives for nutritional applications requiring efficient protein utilization, particularly in sports nutrition, clinical nutrition, and elderly care products, where protein digestibility directly affects therapeutic outcomes.

5.3.11 Water and oil holding capacity

WHC and OHC are terms used to describe the amount of water or oil that a gram of protein can hold, representing crucial functional properties that influence texture, mouthfeel, and processing characteristics in food applications, such as baked goods, meat analogs, and emulsion-based

products. The OHC results (Table 5.3) showed significant variations across lupin protein fractions, with blue lupin albumin achieving the highest OHC (11.61 g/g), followed by white lupin glutelin (10.70 g/g) and blue lupin glutelin (10.55 g/g). The superior OHC of blue lupin albumin fractions compared to other fractions aligns with findings reported for other legume proteins, such as pea and fava bean, where structural flexibility and molecular weight were shown to enhance lipid-binding capacity (Rajpurohit et al., 2024). Under both reducing (Figure 5.1A, lanes d and e) and non-reducing conditions (Figure 5.1B, lanes l and m), albumin fractions displayed prominent bands in the 10-75 kDa range, with particularly intense bands at approximately 20-37 kDa and lower molecular weight regions. Lower-molecular-weight proteins typically have higher surface area to volume ratios, enabling more extensive interactions with oil droplets. Additionally, the smaller polypeptides can more readily unfold and rearrange at oil-water interfaces, exposing hydrophobic amino acid residues that facilitate oil binding. The electrophoresis patterns confirm that albumin's superior oil-holding capacity directly correlates with its abundance of low-molecular-weight peptides, which provide greater interfacial flexibility and oil encapsulation efficiency than the larger, more rigid protein assemblies found in the globulin and glutelin fractions. In addition, the high presence of alkyl and aromatic functional groups in the BLAlb fraction could also be attributed to the high OHC of the BLAlb fraction owing to an increase in hydrophobic interactions. Interestingly, despite the globulin fractions showing moderate surface hydrophobicity (609.05-641.39), this did not translate to superior OHC values, emphasizing that their compact protein conformation might have played a more crucial role than surface properties alone (Liu et al., 2022). The high OHC observed in blue lupin albumin (11.61 g/g) substantially exceeds values reported for conventional plant proteins like soybean (1.8-3.2 g/g) and pea (1.2-2.8 g/g), suggesting significant potential for applications requiring enhanced oil binding such as

meat analogs, bakery products, and salad dressings where oil retention is critical (Tiruneh et al., 2025). There was no significant difference in the OHC between white and blue lupin glutelins; this result correlates with their high surface hydrophobicity, which could be attributed to protein unfolding during alkaline extraction (Sun et al., 2019).

The WHC of proteins, which indicates their ability to retain water without dissolution, is also essential in determining the suitability of food formulations that require moisture retention, for example, the unappealing phase separation in yoghurt after storage, showing poor WHC (Rajpurohit et al., 2024). Glutelin fractions exhibited the highest WHC (1.26-3.10 g/g), significantly exceeding albumin (0.36-0.79 g/g) and globulin (1.14-1.56 g/g) fractions, demonstrating superior water binding capacity despite their reduced solubility characteristics. These WHC patterns appear to be inversely related to solubility characteristics, with proteins with reduced aqueous solubility exhibiting enhanced WHC. Thus, the albumin fractions, which were water extracts with high solubility, displayed the lowest WHC. This enhanced WHC in glutelin correlates with extensive protein unfolding during alkaline extraction, as supported by the CD results at pH 7, which indicate a high beta-sheet and unordered structure content, resulting in expanded molecular conformations with increased surface area and exposed polar amino acid residues capable of hydrogen bonding with water molecules. The combination of high surface hydrophobicity, thermal stability, and superior WHC positions in glutelin fractions is particularly suitable for processed food applications that require moisture retention under thermal processing conditions. Overall, the complementary high OHC and WHC characteristics of different lupin fractions enable targeted selection based on specific food system requirements, with glutelin fractions offering superior water binding for moisture-critical applications, and albumin fractions providing enhanced oil retention for lipid-rich formulations.

5.3.12 Foaming properties

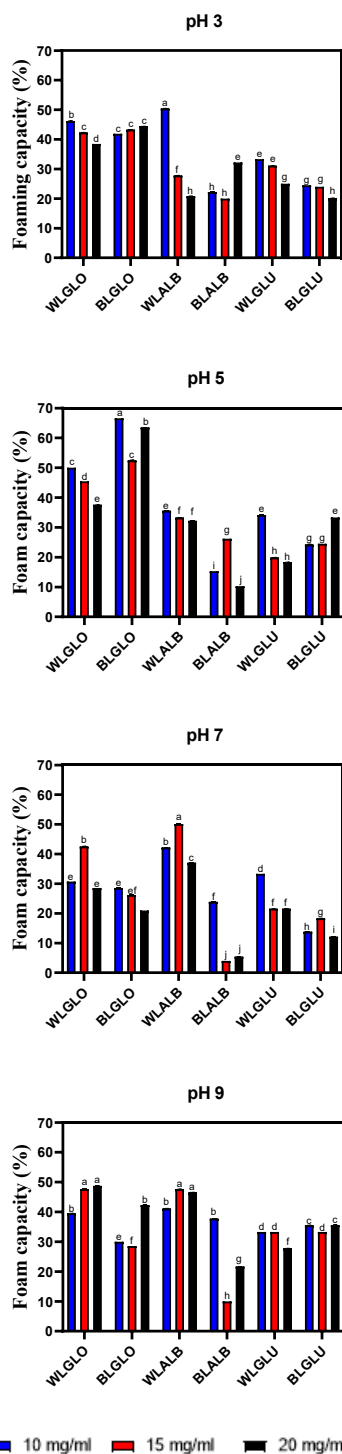
The foaming capacity (FC) of lupin protein fractions revealed distinct pH and protein concentration dependencies that reflect the complex interplay between protein structural flexibility, surface activity, and air-water interfacial properties. Effective foam formation requires proteins to rapidly interact with the air-water interface, undergo controlled unfolding to expose both hydrophobic and hydrophilic regions, and establish continuous interfacial films capable of entrapping air bubbles (Ma et al., 2024). The FC results (Fig. 5.5A) showed that both lupin globulin fractions displayed superior foaming capacities at pH 3 and 5 (>40%), significantly outperforming the albumin and glutelin fractions. The high foaming capacity of the globulin fractions despite their low protein solubility can be attributed to the partial unfolding and exposure of hydrophobic amino acid residues at acidic pH and the formation of small aggregates that can serve as anchors at the air-water interface to create thicker and more cohesive films (Othmeni et al., 2024). BLGlu had the lowest foaming capacity at pH 7, which may be due to its compact, highly ordered structure and low solubility at neutral pH. The tight conformation restricts the unfolding and exposure of hydrophobic residues, reducing their ability to rapidly adsorb and stabilize air–water interfaces. Additionally, glutelins typically have high molecular weights and form large insoluble aggregates, further hindering their interaction with the air-water interface. At higher protein concentrations (15–20 mg/mL), BLA1b exhibited a reduction in foaming capacity (<5%), which is atypical for albumins that are usually highly soluble and surface-active at neutral pH. This concentration-dependent decline was specific to albumins and reflects their distinctive structural features. Although albumins do not possess the highest total free sulfhydryl (SH) content among the protein fractions (Table 5.3), their SH contents (WLA1b: 27.87 $\mu\text{mol/g}$; BLA1b: 17.33 $\mu\text{mol/g}$) are highly exposed and chemically accessible due to the proteins' small size and flexible polypeptide structure (Figure 5.1). At low concentrations, this structural flexibility promotes rapid diffusion and

interfacial adsorption, supporting foam formation. However, as concentration increases, the exposed SH groups facilitate thiol–disulfide exchange reactions, while the flexible chains undergo molecular entanglement. Together, these processes accelerate aggregation in the bulk phase, reducing the pool of soluble, surface-active species and thereby diminishing foaming capacity. In contrast, globulin fractions do not exhibit this concentration-induced loss of function, even though they contain higher total free SH levels. Their SH groups may be buried mainly within compact, conformationally stable quaternary structures, limiting their reactivity. Furthermore, their rigid architecture reduces susceptibility to concentration-driven unfolding or entanglement. As a result, the decline in foaming at high concentrations may be unique to albumins, arising from the interplay among their high solubility, structural flexibility, and the accessibility—not the quantity—of their SH group (Burgos-Díaz et al., 2016). At pH 9, there was a general improvement in FC across all protein fractions, likely due to enhanced solubility and electrostatic repulsion, which facilitated better spreading and rearrangement at the interface. The FC results are similar to those reported by previous authors for other legume proteins (Othmeni et al., 2024; Van de Vondel et al., 2023; Yang et al., 2022).

The foam stability (FS) emphasizes the role of interfacial film strength and protein-protein interactions in foam formation (Fig. 5.5B). BLGlo and WLGLo displayed moderate-to-high foam stability at pH 3 (~45-80%), with peak values observed for BLGlo at 10 mg/mL. These results suggest that globulins form relatively cohesive films that resist drainage and bubble collapse at low pH values. At pH 5, despite BLGlo having the highest foaming capacity, the foam formed was not stable, recording values as low as 10% at 20 mg/mL. Similarly, albumin fractions (WLA1b and BLA1b), although exhibiting high initial foaming capacity, showed lower stability, possibly because of their lower propensity for film reinforcement (Ma et al., 2024). BLA1b fractions at 20

mg/mL consistently displayed poor FS at pH 3, 7, and 9, likely because the interfacial film formed is thin and less elastic, unstable, and collapses readily (Völz et al., 2021). Interestingly, FS improved for WLGLu and BLGLu at pH 9, particularly at higher concentrations (20 mg/mL), possibly due to enhanced partial unfolding of proteins and increased net charge under alkaline conditions, which reduced the propensity for coalescence of the air bubbles. Species differences were subtle but varied, with blue lupin protein fractions showing marginally superior stability, particularly at pH 3 and 9, and the white lupin fractions showing marginally better stability at pH 5 and 7, making all the fractions particularly suitable for applications requiring foam stability, such as aerated food products and specialty beverages.

A



B

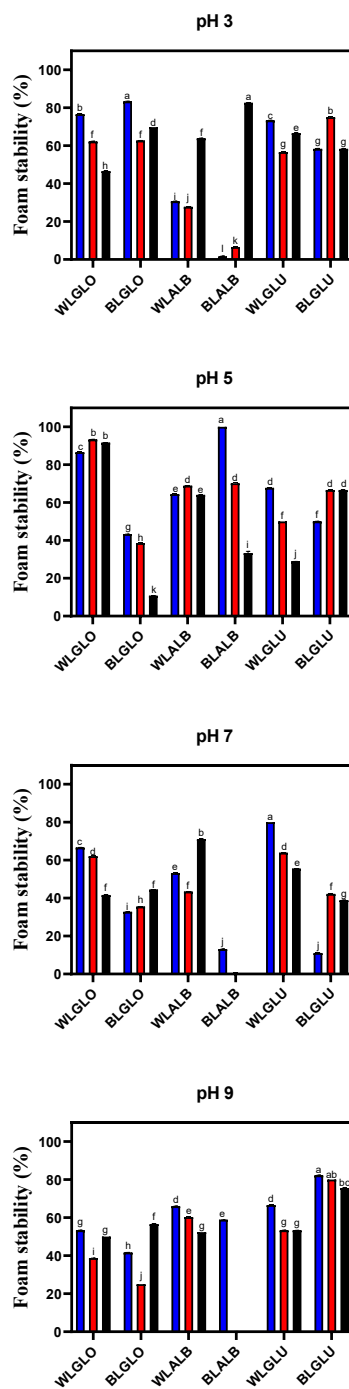


FIGURE 5.5 (A) Effect of pH and protein concentration on the foaming capacity of blue and white lupin albumin, globulin, and glutelin fractions. (B). Effect of pH and protein concentration on the foam stability of blue and white lupin albumin, globulin, and glutelin fractions.

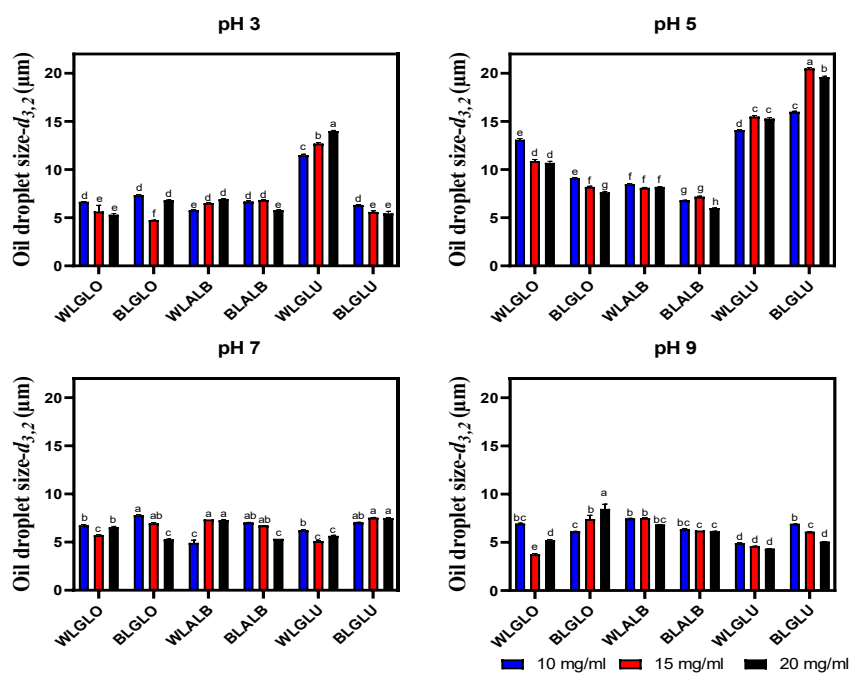
5.3.13 Emulsion formation and stability

The emulsifying capacity of lupin protein fractions demonstrates complex pH and protein concentration dependencies that reflect the intricate relationship between protein structure and interfacial activity. The ability of proteins to form stable oil-in-water emulsions depends fundamentally on their capacity to interact with the oil-water interface, partially unfold to expose both hydrophobic and hydrophilic regions, and form cohesive interfacial films around oil droplets to prevent coalescence (Zhang et al., 2025). At pH 3, all lupin protein fractions had small oil droplet sizes ($\sim 4\text{--}7\ \mu\text{m}$), with the exception of WLGLu fractions, which showed a larger oil droplet size ($\sim 12\text{--}15\ \mu\text{m}$). The small oil droplet sizes observed for these fractions may be due to their optimal surface activity and better solubility at these pH values, which promotes rapid adsorption to the oil–water interface and efficient droplet coverage (Tian et al., 2020). The larger oil droplet size displayed by WLGLu is correlated with the low solubility and far-UV results obtained for this fraction at pH 3. At pH 5, both lupin albumin fractions emerged as superior emulsifiers, producing oil droplets in the $6\text{--}8\ \mu\text{m}$ range compared to the $10\text{--}20\ \mu\text{m}$ for other fractions. This superior emulsifying capacity correlates with the moderate structural changes observed at pH 5, where albumins maintain good solubility and partial secondary structure unfolding, which exposes sufficient hydrophobic regions for effective oil-water interfacial activity (Osemwota et al., 2022). pH, rather than protein concentration, had a greater effect on the emulsifying properties of the lupin protein fractions. The effects of protein concentration were most pronounced at pH 5, where increasing protein levels from 10 to 20 mg/mL resulted in a decrease in the oil droplet size for the globulin and albumin fractions, suggesting optimal protein availability for interfacial coverage. At pH 7 and pH 9, all lupin protein fractions showed consistently good emulsifying properties ($\sim 4\text{--}8\ \mu\text{m}$), which aligns with the structural stability observed in CD and fluorescence analyses, where they maintained relatively low fluorescence intensities and preserved secondary structures,

providing the conformational flexibility necessary for effective interfacial adsorption without excessive aggregation that would impair emulsion quality (Cabrita et al., 2023). These pH-dependent emulsifying properties of lupin protein fractions are consistent with earlier studies on legume proteins, such as pea, chickpea, faba bean and lentil, where protein solubility, secondary structure analysis, and surface hydrophobicity at different pH levels enhanced interfacial behavior (Karaca et al., 2011; Liang & Tang, 2013).

The short-term emulsion stability results (Figure 5.6B) revealed minimal variation across most protein fractions, pH levels, and protein concentrations, except at pH 9, where a notable difference was observed. At pH 3, 5, and 7, all protein fractions maintained high emulsion stability (>85%), regardless of the protein concentration, suggesting that once emulsions were formed, the interfacial film was sufficiently robust to prevent coalescence over time. However, at pH 9, while all protein fractions still displayed high emulsion stability, BLGLo showed a sharp decline, particularly at lower concentrations (15–20 mg/mL). The reduced stability of BLGLo could be attributed to excessive net charge or rearrangement of the polypeptide chains at alkaline pH, resulting in less cohesive interfacial films or phase separation. This is evident from the secondary structure analysis, where aggregation-prone beta sheets predominated at pH 9 for BLGLo. This trend highlights the influence of structural differences between the white and blue lupin globulin fractions on the stability of interfacial films. The high emulsion stability across a wide pH range for lupin protein fractions is an indication of their potential as promising emulsifiers in food systems that require a broad pH range. Similar findings were reported by Östbring et al. (2021), where rapeseed proteins demonstrated pH-dependent emulsion stability linked to their molecular flexibility and aggregation behavior.

A



B

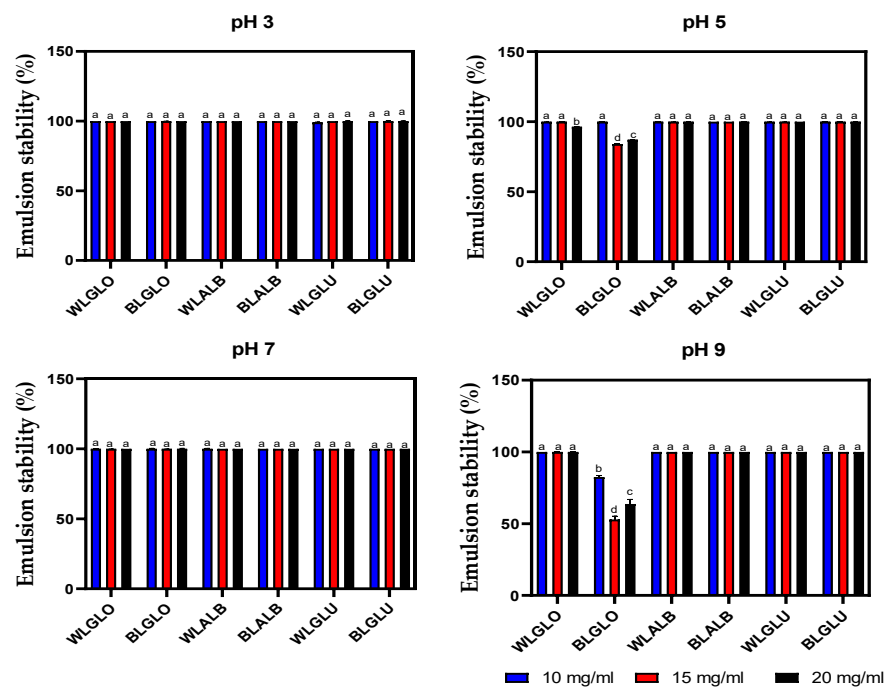


FIGURE 5.6 (A) Effect of pH and protein concentration on the mean oil droplet sizes ($d_{3,2}$) of blue and white lupin albumin, globulin, and glutelin fractions. (B). Effect of pH and protein concentration on the emulsion stability of blue and white lupin albumin, globulin, and glutelin fractions.

5.4 CONCLUSIONS

The lupin species source and protein fraction type significantly affected the conformational state and functional performance of the proteins. Species variation significantly affected the content of isolated fractions, with white lupin favoring conventional globulin accumulation, whereas blue lupin exhibited a more balanced globulin-glutelin distribution. Although legume proteins are typically limited in amino acids like methionine and cysteine, the WLGl fraction is not limited in these amino acids, which indicate an advantage as a nutritional ingredient. The blue lupin protein fractions consistently exhibited higher fluorescence intensities than the white lupin protein fractions across all pH conditions, indicating more open and flexible protein conformations. The SH content had a direct correlation with the interfacial properties of WLGl, which can be due to the ability of free SH groups to rearrange and interact at the oil-water and air-water interfaces, facilitating the absorption of proteins more effectively, thereby stabilizing emulsions and foams. Similarly, the high level of disulfide cross-linking in white lupin albumin fractions could enhance thermal stability and potential use in the formulation of heat-treated food products. The strong effects of pH on emulsifying and foaming properties provide an opportunity for food product developers to manipulate the protein environment in achieving desired product characteristics. Each lupin protein fraction displayed unique properties that can be leveraged as food ingredients, particularly in the formulation of various commercial products across a range of pH and thermal conditions. These findings provide valuable information not only for lupin growers for breeding varieties with optimum functional characteristics, but also for food ingredient manufacturers and consumer companies to select appropriate lupin protein fractions based on specific application requirements.

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AUTHORSHIP

R.E.A Conceived, designed, supervised, reviewed, and edited the manuscript's final draft, S.C. carried out the research, analyzed the data, and wrote the first draft of the manuscript.

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TRANSITION STATEMENT

In chapter 5 (manuscript 4), both lupin species and protein fraction type significantly influence the conformational state and techno-functional performance of lupin proteins. Fractionation of white and blue lupin protein isolates into their albumin, globulin, and glutelin constituents revealed that white lupin appeared to favor globulin accumulation, whereas blue lupin exhibits a more balanced globulin–glutelin distribution. Blue lupin fractions consistently displayed higher intrinsic fluorescence intensities, suggesting a more exposed tertiary structure and potentially greater conformational flexibility, while white lupin globulin stood out for its relatively balanced sulfur-containing amino acids and its high free sulfhydryl content, which correlated with superior interfacial properties. These fraction-level insights confirmed that the isolate-level differences observed in Chapters 3 and 4 arise from distinct contributions of individual protein classes. However, the Osborne solubility-based fractionation employed in Chapter 5 groups proteins by extraction behavior rather than by molecular identity. Each fraction—particularly the globulin fraction—still contains a mixture of structurally distinct components, most notably the 7S vicilins and 11S legumins, which differ fundamentally in quaternary structure, disulfide bonding, and molecular flexibility. To achieve a finer resolution of structure–function relationships, Chapter 6 (Manuscript 5) advances the fractionation strategy by separating the globulin fraction of both lupin species into vicilin and legumin sub-fractions using differential pH-based fractionation. This approach isolates the two dominant storage protein classes and enables a direct comparison of their physicochemical, conformational, and functional properties, providing the molecular-level explanations needed to guide the selection of specific lupin protein ingredients for targeted food applications.

CHAPTER SIX

MANUSCRIPT FIVE

STRUCTURAL, PHYSICOCHEMICAL, AND FUNCTIONAL PROPERTIES OF WHITE AND BLUE LUPIN VICILIN AND LEGUMIN FRACTIONS

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ABSTRACT

The aim of this study was to isolate the vicilin and legumin protein fractions of lupin seeds and determine their physicochemical and functional properties. White lupin vicilin (WLVL) exhibited a significantly higher methionine + cysteine content (2.92 g/100 g protein) than the legumin fractions, with a corresponding amino acid score of 127%. Blue lupin vicilin (BLVL) exhibited high surface hydrophobicity (459.12), whereas the blue lupin legumin (BLLEG) fraction had a high least gelation concentration (18%). All fractions exhibited U-shaped solubility curves, with minimum values at pH 5, while BLLEG showed superior in vitro protein digestibility (87.47%). BLVL demonstrated high emulsifying properties across all pH values, maintaining small droplet sizes (7-9 μm) and high stability (95-100%). BLVL also exhibited superior foaming capacity (75-85%), whereas WLVL showed excellent foam stability (65-80%). These findings revealed significant species-specific and fraction-specific differences in lupin globulins, with BLVL emerging as a promising ingredient for food applications.

Keywords

Lupin, vicilin, legumin, protein digestibility, foaming, emulsion, protein structure

6.1 INTRODUCTION

Legume seeds are one of the most important sources of plant proteins worldwide and have significant nutritional, economic, and environmental benefits. Among legumes, lupins (*Lupinus* spp.) have gained increasing attention because of their high protein content (up to 40%), favorable amino acid profile, low content of anti-nutritional factors, and high dietary fiber content, while being naturally low in starch when compared to other legumes, such as soybeans and peas (Chukwuejim et al., 2023; Duranti et al., 2008). Among the four agroeconomic species of lupin, white lupin (*Lupinus albus*) and blue lupin (*Lupinus angustifolius*) are the most widely cultivated species, especially in regions with temperate climates where they serve as sustainable alternatives to imported soybeans (Lucas et al., 2015). Despite their potential, lupins remain underutilized and have historically been used more for animal feed than for human food (Vogelsang-O'Dwyer et al., 2020). The nutritional and functional properties of lupin proteins are primarily determined by their major storage proteins: vicilins (7S globulins) and legumins (11S globulins), which together constitute approximately 80% of the total seed proteins (Huamaní-Perales et al., 2024; Shrestha et al., 2021).

The structural and functional properties of the vicilin and legumin fractions have been extensively studied in other legume seeds (Chang et al., 2022; Oluwajuyitan & Aluko, 2024). Vicilins (7S) are trimeric proteins with molecular weights ranging from 150 to 190 kDa and are characterized by their high solubility and emulsifying properties (Di Francesco et al., 2024). These proteins have been identified as major lupin allergens, with potential cross-reactivity to peanut allergens (Zhang et al., 2023). Vicilins typically lack disulfide bonds and exhibit greater structural flexibility than legumins. In contrast, legumins (11S) are hexameric proteins with molecular weights between 300 and 400 kDa, containing disulfide-linked acidic and basic subunits that contribute to their thermal stability and gelation properties (Boukid & Pasqualone, 2022; Devkota et al., 2023). The isolation

method significantly influences the protein composition, yield, and functionality of vicilin and legumin fractions (Chang et al., 2022). Different extraction protocols yield distinct protein profiles: alkaline extraction-isoelectric precipitation (AE-IEP) predominantly extracts globulins from pulses, such as peas, fava beans, lupins, and chickpeas (Chukwuejim & Aluko, 2025; Karaca et al., 2011), whereas salt extraction produces globulin-albumin mixtures (Tanger et al., 2022). Salt-extracted protein isolates demonstrate superior functionalities, including solubility, foaming, and emulsifying properties, owing to the presence of albumins, which have higher solubility because of their smaller molecular weight (Meijers et al., 2023; Sha & Xiong, 2022). However, most research works on the structural and functional properties of pulse proteins have focused more on whole protein isolates or globulins rather than the role of individual protein fractions (vicilin and legumins) (Chukwuejim & Aluko, 2025; Gao et al., 2020; Vogelsang-O'Dwyer et al., 2020). Among studies that compared the functional properties of pulse vicilin and legumins, researchers found that vicilin from pea and chickpea exhibited superior solubility and emulsification due to its smaller molecular weight and lower disulfide bond content than legumin (Chang et al., 2022). Another study reported better emulsifying properties for fava bean legumin compared to vicilin (Oluwajuyitan & Aluko, 2024). These discrepancies in the functional properties of vicilin and legumin in other pulses necessitate an investigation into the characteristics of vicilin and legumin from lupin species.

Despite the growing interest in lupin proteins as functional ingredients in food formulations, there remains a significant knowledge gap regarding the comparative structural and functional characteristics of the vicilin and legumin fractions from different lupin species. This gap limits the optimization of lupin protein applications in food systems, thus preventing full exploitation of their potential as sustainable plant protein sources. The present study aims to address this gap by

conducting a comparative analysis of white and blue lupin vicilin and legumin fractions. The work hypothesized that species-specific differences in amino acid composition and surface properties between white and blue lupin vicilin and legumin fractions would result in distinct functional properties, including solubility, emulsification, and foaming properties. Furthermore, we hypothesized that the legumin fraction would show enhanced gelation properties compared to vicilin due to their higher molecular weight and different subunit compositions. In other words, the inherent differences in protein composition and structure between white and blue lupin will translate into distinguishable functional profiles for vicilin and legumin, emphasizing that species selection is a critical factor in the utilization of these plant proteins in food systems. These differences can be strategically exploited to develop tailored functional ingredients with enhanced technological properties for specific food applications. Therefore, the objectives of this study were to isolate and characterize vicilin and legumin fractions from white and blue lupin seeds, compare their structural and functional properties, and establish a relationship between these properties and the influence on their behavior in food systems. These objectives will provide a comprehensive overview of species-specific differences between white and blue lupin protein fractions, supporting their targeted utilization in next-generation plant protein ingredients.

6.2 MATERIALS AND METHODS

6.2.1 Materials

The Lupin Platform Inc. (Calgary, AB, Canada) donated blue (*Lupinus angustifolius* ‘Boregine’) and white (*Lupinus albus* ‘Dieta’) lupin seeds. All experimental work was conducted using high-purity analytical grade reagents and chemicals obtained from either Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific Company (Oakville, ON, Canada). Solutions and preparations were prepared using laboratory-grade double-distilled water (DDW).

6.2.2 Methods

6.2.2.1 Protein extraction and separation into vicilin and legumin fractions

Lupin vicilin and legumin were extracted following the methodology described by Chang et al. (2022) without modification. Lupin seeds were processed into a fine powder using a Retsch ZM200 ultracentrifuge mill at 14,000 rpm with a 0.5 mm ring sieve attachment. The lupin flour obtained was defatted using 1000 mL of acetone for every 100 g of flour. The solvent/flour mixture was stirred for 2 h, after which the liquid phase was discarded. This procedure was repeated twice. The residue obtained after the third acetone extraction was allowed to dry in a ventilated fume cupboard for 12 h. The defatted lupin flour (45.25% and 41.72% protein content for white and blue, respectively) was dispersed in double-distilled water (DDW) at a ratio of 1:15 (w/v), adjusted to pH 9.0 with 1 M sodium hydroxide, stirred for 2 h, and then centrifuged ($5600 \times g$ for 30 min at 4 °C). The supernatant was filtered through Whatman filter paper and then acidified to pH 4.5 using 1 M hydrochloric acid. After stirring for 2 h, the mixture was centrifuged at $5600 \times g$ for 30 min at 4 °C, the supernatant discarded and the precipitate resuspended in 800 mL of 0.2 M sodium borate buffer containing 0.5 M sodium chloride. After stirring for 2 h, the pH was lowered to 4.5 using 6 M glacial acetic acid, followed by centrifugation ($5600 \times g$ for 30 min at 4 °C). The supernatant was collected, neutralized to pH 7.0 with 1 M sodium hydroxide, and dialyzed against deionized water for 48 h to isolate the vicilin fraction. For legumin isolation, the precipitate was redispersed in 200 mL of DDW, stirred for 2 h, and dialyzed against deionized water for 12 h. All dialysis procedures were performed with continuous stirring at 4 °C using a SpectraPor dialysis membrane (6–8 kDa molecular weight cut-off), with the water changed three times daily. Following dialysis, the protein fractions were freeze-dried and stored in sealed containers at -20 °C. The protein content was assessed using the modified Lowry method (Markwell et al., 1978),

with bovine serum albumin serving as the standard. The gross yield was calculated using the formula:

$$\text{Gross yield (\%)} = \frac{\text{Weight of freeze – dried protein fraction (g)}}{\text{Weight of defatted lupin flour (g)}} \times 100 \quad (1)$$

The protein yield (recovery) was calculated using the formula:

$$\begin{aligned} &\text{Protein Yield (\%)} \\ &= \frac{\text{freeze – dried protein fraction weight (g)} \times \text{protein content (\%)}}{\text{Weight of defatted lupin flour (g)} \times \text{protein content (\%)}} \times 100 \quad (2) \end{aligned}$$

6.2.2.2 Amino acid composition

The amino acid profile of lupin protein fractions was determined using Shimadzu Nexara X2 high-performance liquid chromatography (Shimadzu Corp., Kyoto, Japan) with a pico-tag column after the samples underwent acid hydrolysis with 6 M HCl for 24 h. For the analysis of sulfur-containing amino acids (cysteine and methionine), samples were subjected to performic acid oxidation prior to analysis, while tryptophan content was determined following alkaline hydrolysis of the protein fractions. After hydrolysis, derivatization of amino acids was achieved by mixing 70 µL of Waters AccQ-tag buffer with 10 µL of the sample or standard and 20 µL of AccQ-tag reagent followed by incubation at 55°C for 10 min. Separation of regular amino acids was carried out on the AccQ-tag Ultra column using a gradient of Waters AccQTag buffers A and B over 17 min at 0.7 mL/min flow rate and 51°C temperature with UV detection at 260 nm. Cysteine and methionine were determined at 40°C and 60°C, respectively, using a 0.4 mL/min flow rate over 30 min with fluorescence detection at 266 nm excitation and 473 nm emission wavelengths. Tryptophan was analyzed after separation of the alkaline digest on a C18 column (Phenomenex, Torrance, CA,

USA) by isocratic elution (1.0 mL/min flow rate at 28 °C and total run time of 34 min) using a buffer containing 0.3% acetic acid, 0.05% 1,1,1-trichloro-2-methyl-2-propanol, and 5% methanol, which was adjusted to pH 5.0 with ethanolamine. Tryptophan was detected by measuring fluorescence intensity at 280 nm excitation and 356 nm emission wavelengths. The amino acid score was calculated using the FAO/WHO guideline of 2013 for older children, adolescents, and adults (Sá et al., 2024).

6.2.2.3 Gel electrophoresis

White and blue lupin vicilin and legumin fractions were subjected to non-reducing and reducing polyacrylamide gel electrophoresis (PAGE) using the mini-PROTEAN precast TGX gels (4-15%) following the methodology outlined by Osemwota et al. (2021).

6.2.2.4 Size exclusion chromatography

Molecular size distribution was evaluated using an AKTA purifier fast protein liquid chromatography (FPLC) system fitted with a Superdex 75/300 GL column (GE Healthcare Sciences, Montreal, PQ, Canada). For each analysis, a 1 mL sample (20 mg/mL dissolved in 0.05 M sodium phosphate buffer containing 0.15 M NaCl, pH 7.2) was injected and eluted with phosphate buffer (pH 7.2) at a constant flow rate of 0.5 mL/min (isocratic elution). The column was standardized using a series of molecular weight markers: ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), ribonuclease (13.7 kDa), and aprotinin (6.5 kDa) (Alashi et al., 2014).

6.2.2.5 Solubility

Protein solubility was evaluated as described by Nwachukwu & Aluko, (2018). For each determination, 50 mg of white and blue lupin vicilin and legumin was suspended in 5 mL of buffer solutions with a pH range of 3 to 8. After vortexing for 30 s, the suspensions were allowed to

hydrate for 1 h, followed by centrifugation at 5600× g for 30 min. The protein concentration in each supernatant was quantified using the modified Lowry method as described by Markwell et al., (1978). For comparison, the total protein content of each sample was determined using the same analytical method after complete solubilization in 1 M NaOH solution. The percentage protein solubility was calculated using the following formula:

$$PS (\%) = \frac{\text{protein content of fractions at specific pH}}{\text{total protein content of the fraction}} \times 100 \quad (3)$$

6.2.2.6 Zeta potential

The surface charge (zeta potential) of the white and blue lupin vicilin and legumin fractions was measured following the protocol of Asen & Aluko (2023). Measurements were conducted in different buffer systems: 0.1 M acetate buffer (pH 3 and 5), phosphate buffer (pH 7), or Tris-HCl buffer (pH 9) using a Zetasizer nano-Z instrument (Malvern Instruments Ltd., UK). Sample preparation involved dispersing the protein fractions at a 0.1% (w/v) concentration (protein basis) in the appropriate buffer, followed by overnight agitation at 4°C. Prior to analysis, the samples were brought to room temperature, and the insoluble components removed by centrifugation at 2000 × g for 10 min. The zeta potential measurements were performed with automatic voltage selection, and calculations were based on the Smoluchowski model using optical parameters of 1.45 for refractive index and 0.001 for absorption.

6.2.2.7 Particle size distribution

Particle size distribution analysis of aqueous dispersions of the freeze-dried blue and white lupin vicilin and legumin flours was performed using laser diffraction technology with a Mastersizer 3000 (Malvern Instruments Ltd., Worcestershire, UK). Sample preparation involved dispersing the flours in DDW at 1% (w/v) protein concentration in 50 mL centrifuge tubes, and adjusted to pH 7.0 using 1 M NaOH solution. The dispersion was stirred and allowed to hydrate overnight at

4°C. Prior to the measurement, the samples were allowed to equilibrate to room temperature. For the analysis, the optical parameters were configured with a particle refractive index of 1.45, absorption index of 0.1, and dispersant (water) refractive index of 1.33. The protein dispersions were added to the dispersing unit containing ultrapure water until an optimal laser obscuration of 8-12%. The size distribution data are reported as volume-weighted mean diameter (D_{4,3}), surface area-weighted mean diameter (D_{3,2}), and volume percentiles including D_v(10), D_v(50), and D_v(90) (Chukwuejim & Aluko, 2025).

6.2.2.8 Far UV and near UV circular dichroism

Far- and near-UV circular dichroism (CD) spectra were obtained as described by Osemwota et al. (2021) using a J-815 spectrometer (Jasco Corporation, Tokyo, Japan) at a temperature of 25 °C. The far-UV CD spectrum was recorded at 190 - 240 nm with a protein concentration of 2 mg/mL in buffer at different pH values (3, 5, 7, and 9), and analyzed in a quartz cuvette with 0.5 mm path length. The near-UV spectrum was collected at 250 - 320 nm with a protein concentration of 4 mg/mL in buffer at pH 3, 5, 7, and 9, using a 1 mm path length quartz cuvette. Five consecutive scans were averaged, and simultaneous baseline (buffer) subtraction was performed for each sample. The fractions of secondary structure were calculated from the far-UV data using the CONTIN Reference Set 7 secondary structure determination algorithm (optimized for 190–240 nm) as described by Miles et al. (2022) using the DichroWeb online platform (<http://dichroweb.cryst.bbk.ac.uk/html/home.shtml>).

6.2.2.9 Intrinsic fluorescence

Intrinsic fluorescence intensity analysis was performed using an FP-8350 spectrofluorometer (Jasco Co., Japan). Stock solutions were prepared by dispersing each lupin protein fraction at a concentration of 10 mg protein/mL in appropriate buffer systems: 0.1 M acetate buffer (pH 3 and

5), phosphate buffer (pH 7), or Tris-HCl buffer (pH 9). After centrifugation, supernatants were collected and diluted to 0.002% (w/v) with their respective buffers. Samples were excited at 280 nm to stimulate fluorescence from aromatic amino acids (primarily tyrosine and tryptophan), and emission spectra recorded in the range of 300–500 nm. Blank buffer emissions were subtracted from the corresponding sample readings to obtain the protein fluorescence spectra (Aderinola et al., 2020).

6.2.2.10 Surface hydrophobicity

Surface hydrophobicity (S_0) was determined using 8-Anilinonaphthalene-1-sulfonic acid (ANS) as a fluorescent hydrophobic probe (Kato & Nakai, 1980). In the procedure, a 10 mg/mL protein solution was serially diluted to concentrations ranging from 50 to 250 $\mu\text{g/mL}$ in 0.01 M phosphate buffer (pH 7.0). For measurement, 20 μL of ANS solution (8.0 mM prepared in the same phosphate buffer) was added to a 4 mL aliquot of each protein dilution. The fluorescence intensity (FI) was measured using an FP-8350 fluorescence spectrophotometer (Jasco Inc., Tokyo, Japan) at excitation and emission wavelengths of 390 and 470 nm, respectively. The S_0 value was calculated as the linear regression slope obtained by plotting fluorescence intensity against protein concentration.

6.2.2.11 Differential scanning calorimetry

The thermal characteristics of white and blue lupin vicilin and legumin fractions were analyzed using a TA Q100-DSC thermal analyzer (TA Instruments, Newcastle, DE, USA). For each analysis, 15 mg of the protein fractions was weighed and placed in an aluminum pan, which was hermetically sealed. The samples were heated from 40 °C to 140 °C at a constant rate of 10 °C/min, with a sealed empty aluminum pan as the reference. The key thermal parameters—onset temperature (T_o), denaturation temperature (T_d), and enthalpy of denaturation (ΔH)—were

determined using Universal Analysis 2000 software (Version 4.5). Each measurement was conducted in triplicate (Chukwuejim & Aluko, 2025).

6.2.2.12 In vitro protein digestibility

The in vitro protein digestibility of the blue and white lupin vicilin and legumin fractions was evaluated using a method described previously by Hsu et al. (1977). Protein samples were prepared at a concentration of 6.25 mg protein/mL in DDW and adjusted to pH 8 using NaOH (0.1 M) while maintaining a temperature of 37°C under continuous stirring. Enzymatic digestion was initiated by adding 3 mL of a multi-enzyme solution (containing 1.6 mg trypsin, 3.1 mg chymotrypsin, and 1.3 mg peptidase per mL) to 30 mL of each protein suspension. The pH of the reaction mixture was monitored and recorded at 30 s intervals throughout a 10-min digestion period. The protein digestibility percentages were calculated using the regression equation developed by Hsu et al. (1977).

$$\% \text{ Protein digestibility (Y)} = 210.46 - 18.10X_f \quad (4)$$

6.2.2.13 Sulphydryl group content

The sulphydryl (SH) and disulfide bond (SS) content was determined using a previously established methodology (Chukwuejim & Aluko, 2025). Protein fractions (7.5 mg/mL) were dispersed in Tris-Gly buffer (pH 8.0) containing 0.004 M EDTA and 8 M urea and then stirred at room temperature overnight. For free SH content determination, 1 mL of the prepared protein dispersion was combined with 4 mL of Tris-Gly buffer and 0.05 mL of Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)). Following incubation for 20 min at 25°C, absorbance was measured spectrophotometrically at 412 nm. To determine total SH content, 1 mL of protein dispersion was mixed with 4 mL of Tris-Gly buffer and 0.05 mL of 2-mercaptoethanol (ME). This mixture was incubated for 1 h at 25°C, after which ME was removed through three sequential

centrifugation and resuspension cycles. The resulting precipitate was resuspended in 10 mL Tris-Gly buffer, and 0.04 mL of Ellman's reagent was added to 4 mL of this suspension. After 20 min of incubation, absorbance was measured at 412 nm.

6.2.2.14 Least gelation concentration

The least gelation concentration (LGC) of blue and white lupin vicilin and legumin fractions was determined using a previously described method (Malomo & Aluko, 2015). Protein fraction solutions at concentrations ranging from 2% to 20% (w/v, protein basis) were prepared in 0.1 M phosphate buffer pH 7. These solutions were vortexed for 5 min and heated in a water bath at 95°C for 1 h. After heating, the tubes were cooled to room temperature and stored at 4°C for 14 h. The LGC was identified as the minimum protein concentration at which the gel remained stable when the tube was inverted.

6.2.2.15 Water and oil holding capacity

The water and oil-holding capacities were assessed using the method described by Aderinola et al. (2020). For oil holding capacity (OHC) determination, protein fractions at 40 mg/mL were transferred into pre-weighed 15 mL centrifuge tubes, and 5 mL of canola oil added. The mixture was vortexed for 1 min and allowed to stand at room temperature for 30 min. Following centrifugation at $5600 \times g$ for 30 min, excess oil was drained for 15 min, and the tubes were reweighed to calculate the amount of oil retained per gram of protein. The water-holding capacity was determined using the same procedure by substituting the oil with 0.1 M phosphate buffer pH 7 solution.

6.2.2.16 Emulsion formation and stability

Oil-in-water emulsions were prepared following a modified version of the protocol established by Asen & Aluko (2023). Protein fraction solutions at concentrations of 10, 15, or 20 g/L were

formulated in different buffer systems: 0.1 mol/L acetate buffer (pH 3), phosphate buffer (pH 5, 7), and Tris buffer (pH 9). To create the emulsions, 1 mL of refined commercial canola oil was combined with 5 mL of each protein solution and homogenized for 1 minute at 20,000 rpm using a Polytron PT 3100 homogenizer (Kinematica AG, Lucerne, Switzerland) fitted with a 12-mm generator. The surface-weighted mean diameter ($d_{3,2}$) of oil droplets in the emulsions was measured using a Mastersizer 3000 (Malvern Panalytical Ltd., Malvern, UK) with Milli-Q water as the dispersant. Samples were added to approximately 100 mL of water in a small-volume wet sample dispersion unit (Hydro 3000 from Malvern) connected to the instrument under continuous stirring until the appropriate obscuration level was reached. The mean oil droplet size was calculated from five measurements. Emulsion stability was assessed by repeating the measurements after allowing the emulsions to stand undisturbed at room temperature for 30 minutes, with stability calculated using a specified equation.

$$\text{Emulsion stability (\%)} = \frac{\text{Mean oil droplet size at 0 min}}{\text{Mean oil droplet size at 30 min}} \times 100 \quad (5)$$

6.2.2.17 Confocal laser scanning microscopy

Emulsion microstructure analysis was conducted following the protocol established by Tian et al., (2020), employing a Zeiss laser confocal microscope LSM 700 equipped with a Zeiss Imager M2 microscope (Oberkochen, Germany). Freshly prepared emulsion samples were stained with a solution containing both Nile Red (1 mg/mL in ethanol) and fluorescein isothiocyanate (FITC; 1 mg/mL in ethanol) in appropriate quantities. Visualization was performed using specific detection wavelengths: 561 nm to observe Nile Red-stained oil droplets and 418 nm to visualize FITC-labeled proteins.

6.2.2.18 Foaming properties

Foam properties of blue and white lupin vicilin and legumin fractions were evaluated using the method described by Osemwota et al. (2021). Protein dispersions at various concentrations (10, 15, and 20 mg protein/mL) were prepared in different buffer systems: 0.1 M acetate buffer (pH 3 and 5), phosphate buffer (pH 7), or Tris-HCl buffer (pH 9). These mixtures were vortexed for 1 min and allowed to hydrate for 30 min. Foam generation was performed in a 50 mL graduated centrifuge tube using a Polytron PT 3100 homogenizer (Kinematica AG, Lucerne, Switzerland) equipped with a 20 mm shaft, operating at 20,000 rpm for 1 minute. Foaming capacity was determined by measuring the volume of foam produced immediately after homogenization.

Foaming Capacity (FC)

$$= \frac{(\text{volume after homogenization} - \text{volume before homogenization})}{(\text{volume before homogenization})} \times 100 \quad (6)$$

To assess foam stability, the foam volume remaining after 30 min at ambient temperature was measured and calculated as a percentage of the initial foam volume.

6.2.2.19 Statistical analysis

Statistical analyses were conducted in triplicate, with results expressed as mean $\pm$ standard deviation. Analysis of variance was performed using IBM SPSS statistical software (version 28). Duncan's multiple range test was applied to determine statistically significant differences among samples, with significance set at $p < 0.05$.

6.3 RESULTS AND DISCUSSION

6.3.1 Protein extraction and fractionation

The extraction and fractionation efficiency of storage proteins from different lupin species represents a critical parameter that directly influences their commercial viability as alternative plant protein ingredients. Table 6.1 presents the gross yield, protein recovery, and protein content

of the vicilin and legumin fractions isolated from white and blue lupin seeds. Significant differences were observed in the extraction parameters between the protein fractions and lupin species, reflecting their distinct structural characteristics under the applied fractionation conditions. Legumin fractions from both lupin species exhibited substantially higher gross yields (17.33-17.62 g/100 g) compared to vicilin fractions (7.01-8.17 g/100 g), indicating that legumins constitute the predominant storage protein in lupin seeds. This finding aligns with that of Duranti et al. (2008), who identified legumins as the major storage protein components in various lupin species. No significant difference was observed in the gross yield of legumin fractions between white and blue lupin species ($p > 0.05$), suggesting similar legumin contents across these species. However, white lupin vicilin (WLVL) showed significantly higher gross yield (8.17 g/100 g) compared to blue lupin vicilin (BLVL, 7.01 g/100 g), factors such as total protein availability, storage seed protein accumulation, and extraction efficiency may explain the significant difference between WLVL and BLVL. However, based on the protein content of the defatted lupin flours and the resulting protein yield, this difference suggests a variation in total protein availability in both WLVL and BLVL. Blue lupin legumin (BLLEG) demonstrated significantly higher protein recovery (49.93 g/100 g) compared to white lupin legumin (WLLEG, 40.20 g/100 g) ($p < 0.05$), suggesting enhanced extractability of blue lupin legumins under the applied fractionation conditions. Protein content analysis revealed that vicilin fractions exhibited significantly higher protein purity than legumin

Table 6.1 Gross yield, protein recovery, protein content, surface hydrophobicity, thermal, functional, sulfhydryl group, and disulfide linkages of white and blue lupin legumin and vicilin

Parameters	WLVL	BLVL	WLLEG	BLLEG
Gross Yield (g/100g)	8.17 ± 0.51 ^b	7.01 ± 0.13 ^c	17.33 ± 0.57 ^a	17.62 ± 0.23 ^a
Protein recovery (g/100g)	22.81 ± 1.43 ^c	21.17 ± 0.39 ^c	40.20 ± 1.32 ^b	49.93 ± 0.66 ^a
Protein content (% wet basis)	96.99 ± 0.78 ^a	91.73 ± 1.76 ^b	80.68 ± 0.45 ^d	86.13 ± 1.39 ^c
Surface hydrophobicity (S ₀)	315.54 ± 22.7 ^d	459.12 ± 8.07 ^b	513.47 ± 1.68 ^a	353.44 ± 17.33 ^c
<i>Thermal properties</i>				
T _o Onset (°C)	80.56 ± 1.12 ^b	98.75 ± 0.58 ^a	99.17 ± 0.42 ^a	72.17 ± 5.62 ^c
Maximum T _d (°C)	92.61 ± 2.99 ^c	102.45 ± 0.50 ^b	106.17 ± 0.54 ^a	88.15 ± 0.76 ^d
ΔH Area (J/g)	2.77 ± 0.17 ^b	0.32 ± 0.16 ^c	4.02 ± 0.15 ^a	0.32 ± 0.16 ^c
<i>Functional properties</i>				
IVPD (%)	83.94 ± 0.36 ^b	84.65 ± 0.90 ^b	86.20 ± 2.26 ^a	87.47 ± 1.35 ^a
LGC (%)	7.00 ± 0.00 ^b	7.00 ± 0.00 ^b	8.00 ± 0.00 ^b	18.00 ± 0.00 ^a
OHC (g/g)	6.81 ± 0.12 ^a	4.55 ± 0.13 ^b	4.41 ± 0.12 ^b	4.80 ± 0.01 ^b
WHC (g/g)	0.37 ± 0.09 ^b	0.50 ± 0.07 ^b	2.13 ± 0.03 ^a	2.20 ± 0.00 ^a
<i>Sulfhydryl group (SH) and disulfide linkages (SS)</i>				
Free SH (μmol/g)	26.74 ± 0.06 ^a	22.18 ± 0.02 ^b	21.69 ± 0.02 ^b	22.25 ± 0.10 ^b
Total SH (μmol/g)	67.81 ± 4.76 ^b	64.08 ± 6.30 ^c	71.34 ± 2.47 ^a	33.62 ± 5.99 ^d
Disulfide bond (μmol/g)	20.53 ± 2.40 ^b	20.94 ± 3.14 ^b	24.82 ± 1.24 ^a	5.68 ± 2.98 ^c

Data are presented as mean ± standard deviation. Values with different superscripts within the same row are significantly different ($p < 0.05$) as determined by ANOVA and Duncan's multiple range test. WLVL, white lupin vicilin; WLLEG, white lupin legumin; BLVL, blue lupin vicilin; BLLEG, blue lupin legumin.

Table 6.2 Amino acid profile (g/100 g) of white (WL) and blue (BL) lupin vicilin and legumin fractions

Amino acid (AA)	WL Vicilin		BL Vicilin		WL Legumin		BL Legumin	
		AA Score (%)		AA Score (%)		AA Score (%)		AA Score (%)
Indispensable								
Histidine	1.95 ± 0.05 ^a	122 ^γ	2.65 ± 0.29 ^a	126 ^β	1.95 ± 0.07 ^a	122 ^γ	2.31 ± 0.15 ^a	144 ^α
Threonine	2.16 ± 0.04 ^c	86 ^γ	2.10 ± 0.07 ^c	84 ^γ	2.98 ± 0.01 ^a	119 ^α	2.78 ± 0.01 ^b	111 ^β
Lysine	3.38 ± 0.10 ^b	70 ^δ	4.02 ± 0.17 ^a	84 ^β	4.31 ± 0.14 ^a	90 ^α	3.84 ± 0.04 ^{ab}	80 ^γ
Methionine+ Cysteine	2.92 ± 0.02 ^a	127 ^α	1.67 ± 0.04 ^b	73 ^β	1.65 ± 0.02 ^b	72 ^β	1.43 ± 0.01 ^c	62 ^γ
Valine	2.63 ± 0.07 ^b	66 ^β	2.42 ± 0.08 ^b	61 ^γ	3.55 ± 0.03 ^a	89 ^α	3.50 ± 0.02 ^a	88 ^α
Isoleucine	3.77 ± 0.11 ^b	126 ^γ	3.76 ± 0.11 ^b	125 ^γ	4.37 ± 0.02 ^a	146 ^α	3.94 ± 0.01 ^b	131 ^β
Leucine	8.11 ± 0.22 ^a	133 ^α	6.67 ± 0.23 ^b	109 ^γ	7.16 ± 0.03 ^b	117 ^β	6.67 ± 0.09 ^b	109 ^γ
Phenylalanine+ Tyrosine	6.59 ± 0.01 ^b	161 ^γ	6.82 ± 0.13 ^b	166 ^β	8.72 ± 0.02 ^a	213 ^α	6.93 ± 0.10 ^b	169 ^β
Tryptophan	0.23 ± 0.01 ^b	35 ^γ	0.20 ± 0.00 ^b	30 ^δ	0.66 ± 0.05 ^a	100 ^β	0.88 ± 0.12 ^a	133 ^α
Dispensable								
Serine	5.15 ± 0.14 ^a		4.78 ± 0.15 ^a		4.27 ± 0.01 ^b		3.83 ± 0.00 ^c	
Arginine	9.90 ± 0.30 ^b		11.08 ± 0.31 ^a		8.75 ± 0.01 ^c		10.09 ± 0.03 ^b	
Glycine	2.39 ± 0.05 ^d		2.74 ± 0.07 ^c		2.95 ± 0.00 ^b		3.25 ± 0.00 ^a	
Aspartic acid	10.80 ± 0.32 ^a		10.04 ± 0.41 ^{bc}		10.18 ± 0.11 ^{bc}		9.43 ± 0.11 ^c	
Glutamic acid	28.74 ± 0.89 ^a		25.13 ± 0.93 ^b		20.04 ± 0.20 ^c		21.98 ± 0.17 ^c	
Proline	3.13 ± 0.07 ^b		3.12 ± 0.10 ^b		3.73 ± 0.00 ^a		3.80 ± 0.01 ^a	

Alanine	1.80 ± 0.04^b	1.86 ± 0.08^b	2.65 ± 0.03^a	2.73 ± 0.02^a

Data are represented as mean $\pm$ standard deviation. Values with different superscripts (letters and symbols) within the same row are significantly different ($p < 0.05$) as determined by ANOVA and Duncan's multiple range test.

fractions in both lupin species. White lupin vicilin (WLVL) had the highest protein content (96.99%), followed by blue lupin vicilin (BLVL, 91.73%), suggesting that the applied fractionation protocol effectively purified vicilin proteins with minimal non-protein contamination. The significantly higher protein content in white lupin vicilin compared to blue lupin vicilin ($p < 0.05$) indicates potential species-specific differences in vicilin structure or composition that influence purification efficiency. Similarly, blue lupin legumin (BLLEG) contained significantly higher protein content (86.13%) compared to white lupin legumin (WLLEG, 80.68%) ($p < 0.05$). The relatively lower protein content in legumin fractions compared to vicilin fractions may be attributed to the co-extraction of non-protein components, such as phytate or phenolic compounds, which form complexes with legumin proteins owing to their larger molecular size and more complex quaternary structure (Czubinski et al., 2021).

6.3.2 Surface hydrophobicity

Surface hydrophobicity refers to the degree of exposure of non-polar amino acid residues on a protein's molecular surface that preferentially interact with hydrophobic substances while avoiding contact with water. This property significantly influences protein functionality in food systems, particularly affecting emulsification, foaming, and interfacial properties by determining protein-water and protein-lipid interactions. The result from Table 6.1 showed that white lupin legumin (WLLEG) exhibited the highest surface hydrophobicity ($S_0 = 513.47$), whereas the lowest surface hydrophobicity among the fractions was white lupin vicilin (WLVL, 315.54). The high surface hydrophobicity of WLLEG ($S_0=513.47$) can be attributed to its amino acid composition, as shown in Table 6.2. WLLEG exhibited the highest total non-polar amino acid content (35.44 g/100 g protein) among all fractions, and this high amount of hydrophobic amino acids directly contributes to increased surface hydrophobicity through interactions of their non-polar side chains. The high

surface hydrophobicity of WLLEG and BLVL exceeds the values typically reported for other legume protein fractions, including soy glycinin and β -conglycinin, pea legumin and vicilin (over a 100 %), and French bean globulins (Kimura et al., 2008; Meijers et al., 2023). The surface hydrophobicity values correlated with several functional parameters observed in these protein fractions.

6.3.3 Differential scanning calorimetry

The thermal properties of proteins indicate their resistance to denaturation during heat processing, which involves heat treatments such as pasteurization, sterilization, and cooking. The analysis of lupin protein fractions via differential scanning calorimetry is presented in Table 6.1. The onset denaturation temperature (T_o) and maximum denaturation temperature (T_d) showed distinct patterns among the fractions. White lupin legumin (WLLEG) exhibited the highest thermal stability ($T_o = 99.17^\circ\text{C}$, $T_d = 106.17^\circ\text{C}$), followed closely by blue lupin vicilin (BLVL). White lupin vicilin (WLVL) displayed moderate thermal stability ($T_o = 80.56^\circ\text{C}$, $T_d = 92.61^\circ\text{C}$), while blue lupin legumin (BLLEG) showed the lowest resistance to thermal denaturation ($T_o = 72.17^\circ\text{C}$, $T_d = 88.15^\circ\text{C}$). This pattern differs from observations for other legume proteins, where legumin fractions typically demonstrate higher thermal stability than vicilin fractions. For instance, Meng & Ma (2001) reported higher denaturation temperatures for red bean legumin than for pea vicilin, which was attributed to the hexameric structure of legumin and greater disulfide bond content. Similarly, Messian et al. (2015) observed that pea legumin required higher temperatures for denaturation than vicilin fractions. The reversed pattern in blue lupin proteins, where vicilin shows superior thermal stability compared to legumin, suggests unique structural characteristics, such as glycosylation, since sugars are known to have a protective effect on proteins against thermal denaturation (Meng & Ma, 2001). Such differences in thermal properties directly affect protein

functionality in thermally processed food systems, providing essential guidance for selecting appropriate lupin protein fractions for specific food processing applications.

6.3.4 In vitro protein digestibility

The nutritional value of proteins extends beyond their amino acid composition and quantity to include bioavailability after digestion. Protein digestibility is a critical metric for assessing the nutritional quality of protein-based food. For lupin proteins, digestibility may be compromised by several factors, including antinutritional components such as phytic acid and tannins, as well as the complex structural characteristics of plant storage proteins, namely their large molecular size and intricate folded conformations, all of which can impede enzymatic breakdown during digestion. The *in vitro* protein digestibility (IVPD) values of white and blue lupin vicilin and legumin fractions is shown in Table 6.1. All lupin protein fractions exhibited moderate digestibility values, ranging from 83.94% to 87.47%, indicating good nutritional quality and potential as protein sources for human consumption. These values exceed those typically reported for other plant proteins, including soy isolate (70-80%), pea isolates (82%), and faba bean isolate (75-78%), as previously reported (Shi et al., 2022). Blue lupin legumin demonstrated the highest *in vitro* protein digestibility (87.47%), and this may be attributed to the lower disulfide bond content in BLLEG (5.68 $\mu\text{mol/g}$) compared to WLLEG (24.82 $\mu\text{mol/g}$), as shown in Table 6.1. Reduced disulfide cross-linking in BLLEG likely results in a more accessible protein structure for proteolytic enzymes during digestion, enhancing its digestibility. This finding is consistent with the observations of (Wang et al., 2009), who reported an inverse relationship between disulfide bond content and protein digestibility in legume proteins. The high tryptophan content of BLLEG (0.88 g/100 g Table 6.2) could also play a role in the *in vitro* protein digestibility, since tryptophan residues serve as specific cleavage sites for key digestive enzymes used in the Hsu et al., (1977)

method, such as peptidase and chymotrypsin. Conversely, the elevated S_o of BLVL (459.12) corresponded with its high in vitro protein digestibility (84.65%) (Table 6.1), likely due to the increased accessibility of proteolytic enzymes to hydrophobic cleavage sites. Muranyi, et al., (2016) similarly observed correlations between surface hydrophobicity and protein digestibility in various lupin isolates. Comparing protein types, legumin fractions from both lupin species demonstrated superior digestibility compared to their vicilin counterparts, challenging the common assumption that vicilin proteins are more digestible due to their less compact structure. This finding might be explained by the specific amino acid composition of lupin legumins, which could potentially contain sequences that are more susceptible to proteolytic cleavage, despite their more complex quaternary structure.

6.3.5 Sulfhydryl group content

The content and distribution of sulfhydryl groups (SH) and disulfide bonds (SS) in proteins significantly influence their structural stability, functional properties, and behavior during food processing. Table 6.1 presents the free SH, total SH, and disulfide bond contents of the white and blue lupin vicilin and legumin fractions. The free sulfhydryl content showed significant variation among protein fractions, with white lupin vicilin (WLVL) exhibiting the highest value (26.74 $\mu\text{mol/g}$). This finding was expected, given that WLVL displayed the highest methionine and cysteine content, as reported from the amino acid analysis results, suggesting that the distribution and accessibility of cysteine residues may be a result of cleavage during fractionation or the action of endogenous proteases. White lupin legumin (WLLEG) exhibited the highest total SH content (71.34 $\mu\text{mol/g}$) among all fractions, as expected of the general trend observed in other legume proteins, where legumins typically contain more sulfur-containing amino acids than vicilin (Kimura et al., 2008). However, BLLEG has the lowest total sulfhydryl content, highlighting the

unique sulfur amino acid distribution in lupin proteins, which differentiates both lupin species and potentially contributes to their distinct functional properties. White lupin legumin (WLLEG) contained substantially higher disulfide bonds (24.82 $\mu\text{mol/g}$) compared to blue lupin legumin (BLLEG, 5.68 $\mu\text{mol/g}$), representing a greater than four-fold difference. This substantial variation likely arises from the genetic differences that influence protein biosynthesis and folding during seed development. The higher disulfide content in WLLEG may be the reason for its superior thermal stability and distinctive gelation properties compared to BLLEG, as disulfide cross-links provide crucial stabilization during thermal processing and gel network formation (Shen et al., 2025). Vicilin fractions from both lupin species exhibited comparable disulfide bond contents, which were significantly higher than those expected for typical 7S globulins that are generally considered to lack disulfide bonds. Two possibilities may explain the unexpected presence of disulfide bonds in vicilin fractions. First, minor contamination with legumin proteins or protein aggregates may have occurred during the fractionation process (Liang & Tang, 2013). Second, the fractionation procedure may have induced conformational changes that exposed previously buried disulfide groups within the vicilin subunits, making them detectable during analysis (Chang et al., 2022).

6.3.6 Least gelation concentration

The least gelation concentration (LGC) values of the white and blue lupin vicilin and legumin fractions are shown in Table 6.1. Blue lupin legumin (BLLEG) exhibited a high LGC (18%) compared with all other fractions, which showed significantly lower values (7-8 %). This difference suggests that BLLEG forms self-supporting gels at substantially higher concentrations than the other fractions, reflecting fundamental differences in its gelation mechanism or structural characteristics that limit its ability to form extended protein networks. The poor gelation capacity

of BLLEG correlates with its lower surface hydrophobicity (353.44) and reduced disulfide bond content (5.68 $\mu\text{mol/g}$) when compared to the other fractions, as hydrophobic interactions and disulfide cross-linking are critical driving forces in the thermal gelation of globular proteins. Interestingly, no significant difference was observed in the gelation capacity between the white and blue lupin vicilin fractions (both at 7% LGC), despite their considerable differences in surface hydrophobicity and thermal properties. This finding suggests that surface hydrophobicity has a lesser influence on vicilin gelation and protein network formation by this fraction may be more dependent on other factors, such as molecular flexibility or specific subunit interactions. The relatively good gelation properties of vicilin fractions, despite their smaller molecular size, highlight their potential applications in food systems that require gelling at lower protein concentrations. The distinct gelation behaviors among lupin protein fractions have significant implications for their application in food systems. The superior gelation capacity of WLLEG and vicilin fractions makes them potentially valuable ingredients in gel-based food products, such as meat alternatives, tofu-like products, or structured desserts, where gel formation at lower protein concentrations is desirable for economic and textural reasons.

6.3.7 Water and oil holding capacity

The water holding capacity (WHC) and oil holding capacity (OHC) represents the amount of water or oil that can be retained by a specific weight of protein (Ma et al., 2022). These measurements serve as indicators of a protein's ability to immobilize fluid components within food systems, thereby minimizing moisture or fat loss during the manufacturing process and subsequent storage periods (Rajpurohit et al., 2025). The OHC values of the white and blue lupin vicilin and legumin fractions are shown in Table 6.1. White lupin vicilin (WLVL) demonstrated the highest oil holding capacity (6.81 g/g) among all fractions, significantly exceeding the values observed for blue lupin

vicilin (BLVL, 4.55 g/g) and both legumin fractions. The superior lipid-binding ability of WLVL is somewhat unexpected, given its moderate surface hydrophobicity (315.54) when compared to that of BLVL (459.12). This is because higher surface hydrophobicity directly correlates with enhanced oil binding. This anomaly suggests that factors beyond surface hydrophobicity, such as the presence of high levels of non-polar or hydrophobic amino acids, protein conformation, flexibility, or the spatial distribution of hydrophobic patches, may play crucial roles in determining oil-holding capacity (Rajpurohit et al., 2025). The high S_o of WLLEG (513.47) aligns with its superior thermal stability ($T_o = 99.17^\circ\text{C}$) and enhanced water-holding capacity (2.13 g/g), suggesting that exposed hydrophobic patches contribute to temperature resistance and the structured organization of water around the protein. The unique quaternary structure of WLVL, as seen from its higher molecular weight (262 kDa) in SEC analysis (Fig 2) compared to the trimeric structure of BLVL (166 kDa), may create distinct hydrophobic cavities or interfaces that enhance lipid entrapment despite the lower overall surface hydrophobicity. The significant species-specific differences in OHC observed between white and blue lupin vicilins have important implications for their functional applications. The good oil-binding capacity of WLVL makes it particularly suitable for application in fat-reduced meat products, where oil retention during processing and cooking is crucial for juiciness and texture (Chukwuejim & Aluko, 2025). Additionally, WLVL can serve as an effective emulsion stabilizer in products such as plant-based mayonnaise, salad dressings, and sauces, where interactions with lipid components are essential for product stability and sensory attributes (Chutinara et al., 2024). The legumin fractions exhibited higher WHC, with WLLEG showing a higher value than the BLLEG. The approximately 4-fold higher WHC of legumin fractions compared to vicilin fractions indicates fundamental differences in their hydration mechanisms and water-binding sites. This enhanced water-holding ability of legumin

proteins likely stems from their more complex quaternary structure, larger molecular size, and potentially higher proportion of hydrophilic amino acid residues exposed on their surfaces, as suggested by their zeta-potential profiles. The distinct hydration properties of lupin protein fractions have significant implications for their application in food systems. The superior water-holding capacity of legumin fractions makes them particularly valuable ingredients in moisture-sensitive applications, such as meat alternatives, bakery products, and pasta, where water retention during processing and cooking is crucial for texture and sensory attributes. Conversely, the limited WHC of vicilin fractions might be advantageous in formulations where excessive water binding is undesirable, such as crisp-baked goods (Devkota et al., 2024).

6.3.8 Amino acid composition

The amino acid profiles of the white and blue lupin vicilin and legumin fractions (Table 6.2) revealed significant differences in their nutritional value and potential functionality. The amino acid composition not only determines the nutritional quality of proteins but also influences their structural characteristics and techno-functional properties in food systems. This analysis focused on the essential and non-essential amino acids, which are critical determinants of protein quality in plant-based diets. Sulfur-containing amino acids (methionine + cysteine) are typically the limiting amino acids in most legume proteins, making their content especially important for nutritional evaluation. White lupin vicilin (WLVL) exhibited significantly higher methionine + cysteine content (2.92 g/100 g protein) than all other fractions, with a corresponding amino acid score of 127%. This represents a notable nutritional advantage over blue lupin vicilin (BLVL, 1.67 g/100 g protein, score 73%) and both legumin fractions (WLLEG: 1.65 g/100 g, BLLEG: 1.43 g/100 g). These findings contradict the general assumption that legume proteins are universally deficient in sulfur amino acids, suggesting that specific fractions, such as white lupin vicilin, could

serve as superior protein sources in vegetarian and vegan diets. The higher methionine + cysteine content in vicilin fractions from white lupin is particularly important because these amino acids play crucial roles in protein folding, disulfide bond formation, and antioxidant activity through glutathione biosynthesis (Rahman et al., 2021). Tryptophan contents of legumin fractions were significantly higher when compared to the vicilin fractions. Blue lupin legumin (BLLEG) demonstrated the highest tryptophan content (0.88 g/100 g protein, amino acid score of 133%), followed by white lupin legumin (WLLEG, 0.66 g/100 g, amino acid score of 100%). In contrast, vicilin fractions from both species contained substantially lower tryptophan levels (0.20-0.23 g/100 g, corresponding to amino acid scores of 30-35%). This distinct distribution pattern suggests fundamental differences in the evolutionary conservation of tryptophan residues between vicilin and legumin. Tryptophan serves as a precursor for serotonin synthesis and plays critical role in protein structure stabilization through aromatic interactions, making its higher content in legumin fractions nutritionally advantageous (Nunzi et al., 2025). Valine, an essential branched-chain amino acid, showed a similar pattern to tryptophan, with significantly higher contents in legumin fractions compared to the vicilin fractions across both lupin species. White lupin legumin (WLLEG) contained the highest valine content (3.55 g/100 g protein, amino acid score of 89%), closely followed by blue lupin legumin (BLLEG, 3.50 g/100 g, amino acid score of 88%). The vicilin fractions contained moderate valine levels (WLVL: 2.63 g/100 g, BLVL: 2.42 g/100 g) with corresponding amino acid scores of 66% and 61%, respectively. Although the valine content is limited in all fractions, it is particularly relevant for sports nutrition applications, as branched-chain amino acids such as valine are essential for muscle protein synthesis and recovery (Berrazaga et al., 2020). Blue lupin vicilin (BLVL) contained a higher lysine content (4.02 g/100 g protein, amino acid score of 84%) than white lupin vicilin (WLVL, 3.38 g/100 g, amino acid score of 70%),

and the white lupin legumin lysine content (WLLEG, 4.31 g/100 g, amino acid score of 90%) exceeded all other fractions. Lysine is typically a limiting amino acid in cereal-based diets, making lupin proteins, especially white lupin legumin, valuable complementary proteins for improving the nutritional quality of cereal-based food formulations, although lupin fractions did not meet the recommended score. Lysine also contributes to the functional properties of proteins, as lysine residues participate in Maillard reactions during food processing, thereby influencing flavor development and browning (Arsa & Puechkamutr, 2022).

6.3.9 Solubility

The solubility profiles of the white and blue lupin globulin fractions at different pH values (Figure 6.1) revealed distinct patterns that reflect their structural characteristics and potential applications. Protein solubility is a critical functional property that influences other techno-functional attributes such as emulsification, foaming, and gelation capabilities in food systems. All protein fractions exhibited a typical U-shaped solubility curve, with minimum solubility at pH 5, corresponding to their isoelectric points. This behavior aligns with the fundamental principle that proteins have minimal solubility at their isoelectric point due to reduced electrostatic repulsion between molecules, which promotes protein-protein interactions and precipitation (Chukwuejim & Aluko, 2024). Interestingly, white lupin vicilin (WLVL) demonstrated superior solubility at pH 7-8, reaching approximately 90-100% solubility, which is significantly higher than its legumin counterpart (WLLEG). This enhanced solubility of vicilin fractions at pH 7 and 8 can be attributed to their lower molecular weight, absence of disulfide bonds, and higher surface hydrophilicity compared to legumins, as previously reported for pea vicilins (Kimura et al., (2008). Similarly, blue lupin vicilin (BLVL) showed moderate solubility at pH 3 but exhibited a distinctive solubility profile at pH 6, which differed from that of white lupin vicilin. The legumin fractions from both

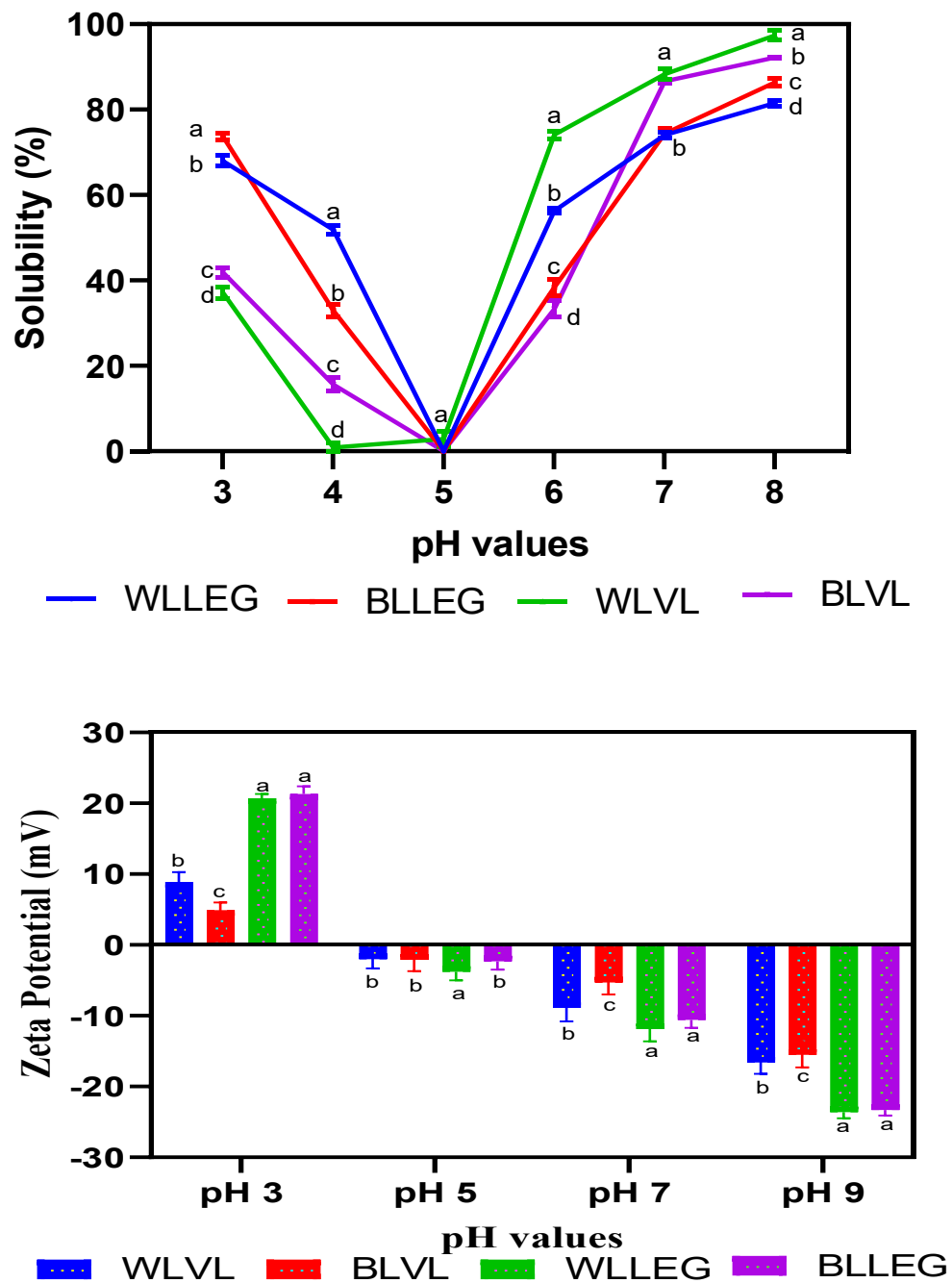


Figure 6.1 Protein solubility profiles and zeta potential of white and blue lupin vicilin (WLVL and BLVL) and legumin (WLLEG and BLLEG) fractions as a function of pH.

lupin species (WLLEG and BLLEG) displayed comparable solubility patterns at pH 3, with BLLEG showing slightly higher solubility (75%) compared to WLLEG (65%). However, a notable species-specific difference was observed at pH 7-8, where WLLEG demonstrated approximately 10% higher solubility than BLLEG. These differences between white and blue lupin globulins are different from the findings of Muranyi, Otto, et al. (2016), who reported similar solubility profiles for total protein isolates from different lupin species, suggesting that fractionation reveals species-specific differences that are not apparent in total protein isolates. The superior solubility of lupin vicilin fractions at pH 7 and 8 suggests potential advantage in food applications that require high protein solubility under neutral to alkaline conditions, such as protein-enriched beverages and dairy alternatives. Conversely, the moderate solubility of lupin legumin fractions at acidic pH indicates their potential suitability for acidic food formulations, such as fruit-based protein drinks and acidified dairy products.

6.3.10 Zeta potential

The zeta potential measurements of lupin globulin fractions across various pH conditions (Figure 6.1) provide valuable insights into their surface charge characteristics, which significantly influence protein-protein and protein-solvent interactions. Zeta potential represents the electrical charge at the shear plane of the protein-solvent interface and serves as a reliable indicator of protein colloidal stability in solution (Lam et al., 2018). At pH 3, both legumin fractions (WLLEG and BLLEG) exhibited strong positive zeta potential values (~20 mV), which were significantly higher than those of their vicilin counterparts (WLVL ~8 mV, BLVL ~4 mV). This substantial difference suggests that legumin fractions possess more exposed positively charged amino acid residues at acidic pH, contributing to stronger electrostatic repulsion between protein molecules, and potentially higher stability against aggregation in acidic environments. As the pH increased from

3 to 9, all the protein fractions showed a progressive shift from positive to negative zeta potential values, crossing the isoelectric point at approximately pH 5, where the zeta potential values approached zero. This transition aligns with the solubility profiles, confirming that the minimal electrostatic repulsion at the isoelectric point correlates with reduced solubility. At neutral and alkaline conditions, all fractions exhibited negative zeta potential values, with the most pronounced negative charges observed at pH 9. Notably, legumin fractions (WLLEG and BLLEG) displayed significantly more negative zeta potential values (-25 to -27 mV) at pH 9 compared to vicilin fractions (WLVL and BLVL, -15 to -20 mV). These differences in surface charge characteristics between vicilin and legumin fractions across both lupin species correspond to their distinct amino acid compositions, particularly the proportion of charged amino acids, as reported by Duranti et al., (2008). The high negative charge of the legumin fractions at alkaline pH suggests their potential superiority in applications requiring electrostatic stabilization of food systems under neutral to alkaline conditions. The species-specific differences in zeta potential were less pronounced compared to the protein-type differences, indicating that protein classification (vicilin vs. legumin) has a more significant impact on surface charge properties than lupin species. These findings align with those of Chang et al. (2022), who reported similar trends in zeta potential profiles for the green pea and chickpea globulin fractions.

6.3.11 Gel electrophoresis

SDS-PAGE analysis of white and blue lupin vicilin and legumin fractions (Figure 6.2A) revealed distinct molecular profiles under reducing and non-reducing conditions. Under reducing conditions, legumin fractions (BLLEG and WLLEG) displayed characteristic 11S globulin patterns with prominent bands at 40-45 kDa (acidic subunits) and 20-25 kDa (basic subunits). White lupin legumin showed different band intensities compared to blue lupin legumin, indicating

species-specific variations in subunit expression or post-translational modifications that may influence their functional properties (Santamaria et al., 2024). Vicilin fractions (BLVL and WLVL) exhibited more complex banding patterns under reducing conditions, with multiple bands between 15-70 kDa typical of 7S globulins. The major bands at 50-55 kDa, 30-35 kDa, and 15-20 kDa correspond to the subunits commonly observed in legume vicilin. The blue lupin vicilin displayed more intense bands at lower molecular weights (15-20 kDa) than the white lupin vicilin, suggesting differences in proteolytic processing during seed development and the fractionation process (He et al., 2021). Under non-reducing conditions, legumin fractions exhibited high molecular weight bands (~60-65 kDa), representing intact subunit pairs linked via disulfide bonds, which confirmed their hexameric structure, as previously reported (Duranti et al., 2008). Some dissociated subunits observed in the non-reduced samples may indicate partial reduction during preparation or endogenous proteolytic activity. The non-reduced vicilin fractions exhibited patterns similar to those of their reduced counterparts, confirming the absence of disulfide bonds in these proteins. This structural characteristic aligns with the trimeric quaternary structure of vicilins, which is stabilized primarily through non-covalent interactions (Kimura et al., 2008). Comparative analysis revealed that white lupin vicilin displayed higher molecular weight distributions compared to blue lupin vicilin, whereas blue lupin legumin showed a more heterogeneous subunit composition than white lupin legumin. These molecular differences, likely arising from genetic variations, contribute to the observed differences in functional properties between white and blue lupin protein fractions, confirming the successful protein fractionation with distinct molecular characteristics relevant for food applications.

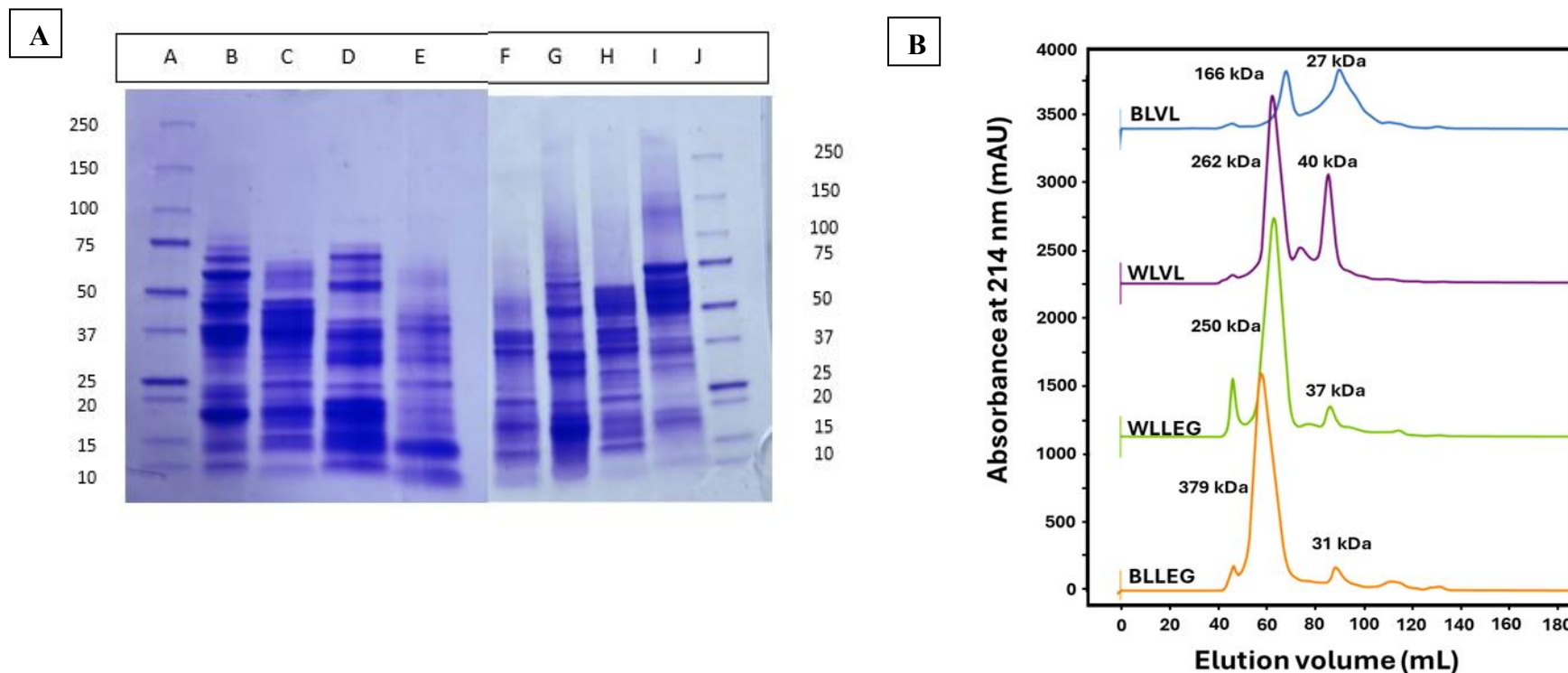


Figure 6.2 (A) SDS PAGE of white and blue lupin vicilin (WLVL and BLVL) and legumin (WLLEG and BLLEG) fractions under reducing and non-reducing conditions. **A**: Standard protein markers (kDa); **B**: reduced BLLEG; **C**: reduced WLLEG; **D**: reduced BLVL; **E**: reduced WLVL; **F**: non-reduced WLVL; **G**: non-reduced BLVL; **H**: non-reduced WLLEG; **I**: non-reduced BLLEG; **J**: Standard protein markers (kDa). Fig. 2(B) Size exclusion chromatography of blue and white lupin vicilin and legumin fractions with UV detection at 214 nm.

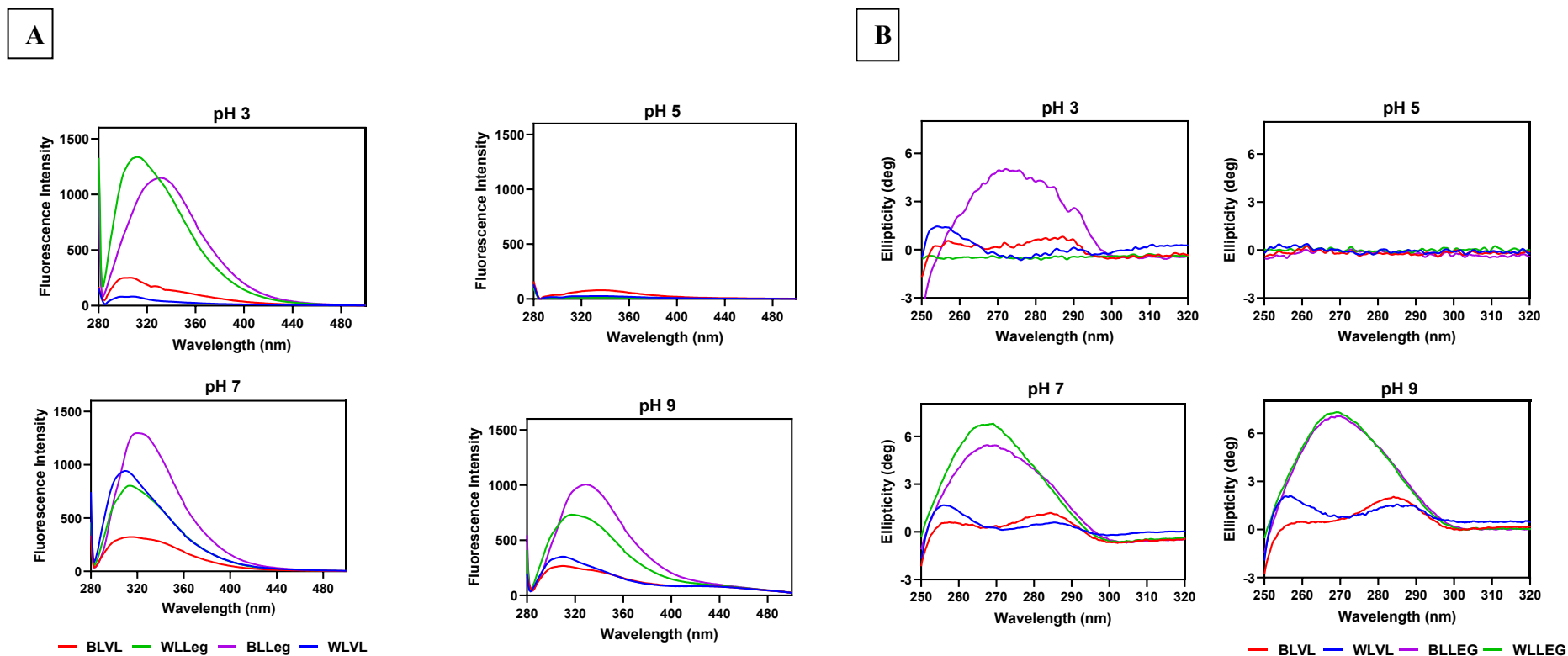


Figure 6.3 (A). Intrinsic fluorescence intensity at different pH values of white and blue lupin fraction. Fig. 3(B). Near UV circular dichroism spectra at different pH values of white and blue lupin fraction. BLVL-blue lupin vicilin; WLVL-white lupin vicilin; WLLEG-white lupin legumin; BLLEG-blue lupin legumin.

6.3.12 Size exclusion chromatography

Size exclusion chromatography analysis of white and blue lupin vicilin and legumin fractions (Figure 6.2B) revealed distinctive native molecular size distributions and quaternary structures in the non-denatured state. This technique provides valuable insights into intact protein assemblies by preserving their native conformations and complementing the SDS-PAGE results. BLVL exhibited two major peaks at 166 kDa and 27 kDa. The 166 kDa peak represents the intact trimeric assembly typical of 7S globulins, while the 27 kDa peak indicates partial dissociation of the trimeric structure into monomeric subunits or proteolytic fragments, suggesting greater structural change in blue lupin vicilin compared to other fractions. WLVL showed a different profile, with major peaks at 262 and 40 kDa. The higher molecular weight of the main peak suggests potential dimerization of trimeric assemblies or associations with other seed components. The 40 kDa peak likely represents monomeric subunits, consistent with the SDS-PAGE findings showing higher molecular weight distributions for WLVL. The legumin fractions exhibited significantly higher molecular weights than the vicilin fractions, confirming their 11S globulin classification. White lupin legumin (WLLEG) displayed major peaks at 250 kDa and 37 kDa. The 250 kDa peak represents the hexameric assembly, which is slightly lower than the typical 300-400 kDa range for 11S globulins, possibly indicating partial dissociation, in vivo endogenous protease digestion, or species-specific variations (Di Francesco et al., 2024). The 37 kDa peak corresponds to the alpha legumin subunit pairs with covalently linked acidic and basic chains. Blue lupin legumin (BLLEG) had the highest molecular weight among all fractions, with peaks at 379 and 31 kDa. The 379 kDa peak confirmed the intact hexameric assembly within the expected range for 11S globulins. The higher molecular weight compared to WLLEG (379 kDa vs. 250 kDa) suggests species-specific differences in the subunit composition or quaternary organization. The smaller proportion of

dissociated subunits indicates greater structural stability in BLLEG, potentially explaining its superior protein recovery during extraction compared to WLLEG. These species-specific differences in quaternary structures have significant implications for protein functionality, as aggregation states directly influence properties such as solubility, gelation, and surface activity.

6.3.13 Particle size distribution

Particle size distribution influences numerous functional properties of protein ingredients, including solubility, dispersibility, water-binding capacity, and sensory attributes in food systems. The microstructural organization of protein particles significantly affects their behavior in complex food matrices and their interactions with other food components (Joshi et al., 2011). Analysis of particle size parameters (Supplementary material Table S1) revealed substantial differences among the lupin protein fractions. White lupin vicilin (WLVL) exhibited markedly larger particles across all measured parameters, with a volume-weighted mean diameter $D_{4,3}$ of 290 μm . Both BLVL and WLLEG showed intermediate particle sizes with BLLEG displaying the smallest particle size distribution ($D_{4,3} = 11.8 \mu\text{m}$), with a notably narrow size range as indicated by its sharp, and uniform peak observed in the distribution curve (Supplementary material Fig.S1). The WLVL particle size distributions differed considerably from those reported for lupin isolates. Vogelsang-O'Dwyer et al. (2020) observed significantly smaller particles in blue lupin protein isolates ($D_{4,3} = 12.1 \mu\text{m}$). The large particles in WLVL exceed the values typically reported for commercial plant protein ingredients, suggesting potential challenges in certain applications where fine particle distribution is critical. The particle size distribution corresponds well with thermal properties, as BLLEG with smaller particles exhibited lower denaturation temperatures ($T_0 = 72.17^\circ\text{C}$), suggesting that reduced particle size may facilitate heat transfer and subsequent unfolding during thermal processing. Conversely, WLVL's large particles likely contribute to its excellent oil-

holding capacity (6.81 g/g) by providing extensive interstitial spaces for oil entrapment, a relationship previously noted by (Rajpurohit et al., 2025) for various food protein systems.

6.3.14 Intrinsic fluorescence

Intrinsic fluorescence spectroscopy provides valuable insights into protein tertiary structure and conformational changes by detecting the emission of aromatic amino acids, particularly tryptophan, which serves as a sensitive indicator of the protein folding state and microenvironment polarity (Liu et al., 2024). The result from Figure 6.3A showed that at acidic pH 3, all protein fractions exhibited strong fluorescence emissions, with legumin fractions (WLLEG and BLLEG) displaying substantially higher intensities (1200-1300 fluorescence units) compared to vicilin fractions (WLVL and BLVL; 150-300 units). This difference may indicate less exposure of aromatic amino acids in legumin proteins to environmental quenchers at acidic pH, which is consistent with their more compact quaternary structures (Hinderink et al., 2021). Notably, BLLEG and WLLEG showed two distinct peaks: a prominent maximum at approximately 340 nm (tryptophan) and a smaller shoulder around 305-310 nm (tyrosine), indicating a folding pattern in which tyrosine and tryptophan residues are sufficiently separated to prevent complete energy transfer (Aderinola et al., 2020). At the isoelectric point (pH 5), all fractions exhibited significant reductions in fluorescence intensity, suggesting an increased exposure of aromatic amino acids to the hydrophilic environment, leading to fluorescence quenching. This structural change directly correlates with the minimum solubility observed at pH 5, confirming that protein aggregation at the isoelectric point alters the microenvironment of aromatic residues. Tyrosine peaks were barely detectable at this pH, indicating closer proximity to tryptophan residues due to protein compaction during aggregation. The fluorescence intensity at pH 5 differs from results obtained by Oluwajuyitan & Aluko (2024) where the high fluorescence intensity at pH 5 was attributed to a

more rigid structure of the legumin fractions. At pH 7, the fluorescence intensity increased substantially for all fractions, and this recovery coincided with increased protein solubility at neutral pH, suggesting unfolding that optimizes both hydrophilic interactions and proper positioning of aromatic residues. Interestingly, vicilin fractions showed slight red shifts in their emission (342-345 nm) compared to legumin fractions (338-340 nm), indicating greater exposure of tryptophan residues to the aqueous environment in the vicilins. At pH 9, all fractions exhibited moderate fluorescence intensity, with slight blue shifts in the maximum wavelength compared to pH 7, particularly for BLLEG. This blue shift, coupled with the maintained fluorescence intensity, suggests structural rearrangements that positioned tryptophan residues in more hydrophobic microenvironments within the protein core (Liu et al., 2024). The pH-dependent fluorescence patterns of lupin globulins is similar to results obtained for pea and phaseolus legume globulins (Sha & Xiong, 2022; Tang & Sun, 2011).

6.3.15 Far-UV and near-UV circular dichroism (CD)

CD spectroscopy is a valuable tool for exploring protein conformation, with far-UV analysis (190-250 nm) revealing secondary structure elements and near-UV (250-320 nm) detection of tertiary structure organization in environments containing aromatic amino acids. This technique is particularly useful for assessing structural changes in proteins influenced by environmental conditions, such as pH, which could affect functional properties in food systems (Hoffmann et al., 2016; Huang et al., 2025). Quantitative analysis of the secondary structure composition (Table 6.3) revealed that the unordered structure predominantly constituted all lupin protein fractions across most pH conditions, with values ranging from 60-70% at pH 3, 7, and 9. This high proportion of unordered structures suggests partial denaturation during protein isolation and fractionation, a phenomenon similar to that observed by Aderinola et al. (2020) for *Moringa oleifera* seed proteins.

Notably, at pH 5 (near the isoelectric point), a significant structural transition occurred, where the β -sheet became the predominant structure for all fractions, with values reaching 45% for WLLEG and 37% for BLLEG. This increase in β -sheet content at pH 5 coincides with the minimum protein solubility and suggests extensive intermolecular hydrogen bonding during protein aggregation, consistent with the previous findings for other legume proteins (Tang & Sun, 2011; Cui et al., 2020). α -Helix content varied significantly with pH and protein type. At pH 3, the α -helix content ranges from 9-16% in all fractions, whereas at pH 7, a sharp increase in α -helix structure was observed for WLVL (21%) and BLVL (16%). This pH-dependent enhancement in the helical structure at neutral pH aligns with optimal protein solubility and functional properties, suggesting favorable electrostatic interactions that promote helix formation. The β -turn structure represented a minor but significant component across all fractions, with higher values generally observed at pH 5 and 7, indicating its essential role in stabilizing protein conformation under these conditions. Near-UV CD spectra (Figure 6.3B) revealed significant tertiary structural differences among lupin globulin fractions across different pH values. At pH 3, blue lupin legumin exhibited the most intense signal, with a characteristic peak around 270-280 nm, indicating a well-defined tertiary structure with tyrosine residues in chiral environments. This result agrees with the high solubility and intense fluorescence obtained at pH 3 for BLLEG. At pH 5, all fractions showed minimal ellipticity, suggesting tertiary structure disruption, concurrent with the observed increase in β -sheet content and minimum solubility. At pH 7 and 9, the tertiary structure was recovered, with blue lupin legumin and white lupin legumin demonstrating more pronounced signals than other fractions, indicating a more organized tertiary structure that correlates with their distinctive functional properties.

Table 6.3 Secondary structure of white and blue lupin vicilin and legumin at different pH values.

WLVL				
pH	3	5	7	9
α-helix	16.75 $\pm$ 0.25 ^c	4.05 $\pm$ 1.15 ^d	21.85 $\pm$ 0.35 ^a	19.15 $\pm$ 0.35 ^b
β-sheet	3.25 $\pm$ 0.65 ^c	35.30 $\pm$ 2.60 ^a	5.85 $\pm$ 3.05 ^b	2.65 $\pm$ 0.85 ^c
Turns	10.60 $\pm$ 1.90 ^c	18.85 $\pm$ 0.25 ^a	12.00 $\pm$ 2.20 ^b	8.90 $\pm$ 2.30 ^d
Unordered	69.40 $\pm$ 2.30 ^a	41.85 $\pm$ 1.65 ^c	60.30 $\pm$ 5.60 ^b	69.30 $\pm$ 2.80 ^a
BLVL				
pH	3	5	7	9
α-helix	9.65 $\pm$ 4.65 ^b	4.00 $\pm$ 1.30 ^c	16.50 $\pm$ 0.30 ^a	9.90 $\pm$ 4.50 ^b
β-sheet	9.05 $\pm$ 3.15 ^b	36.10 $\pm$ 2.20 ^a	3.80 $\pm$ 0.90 ^c	9.55 $\pm$ 2.45 ^b
Turns	14.20 $\pm$ 0.10 ^b	19.15 $\pm$ 0.25 ^a	10.05 $\pm$ 2.15 ^c	13.35 $\pm$ 0.65 ^b
Unordered	67.10 $\pm$ 7.70 ^b	40.75 $\pm$ 1.15 ^c	69.65 $\pm$ 2.75 ^a	67.20 $\pm$ 7.60 ^b
WLLEG				
pH	3	5	7	9
α-helix	9.20 $\pm$ 4.10 ^a	2.00 $\pm$ 0.50 ^c	9.65 $\pm$ 4.45 ^a	7.40 $\pm$ 3.90 ^b
β-sheet	10.55 $\pm$ 3.55 ^c	45.75 $\pm$ 1.85 ^a	9.65 $\pm$ 4.25 ^c	13.00 $\pm$ 4.20 ^b
Turns	14.55 $\pm$ 0.35 ^b	21.00 $\pm$ 0.90 ^a	15.75 $\pm$ 1.85 ^b	15.05 $\pm$ 0.85 ^b
Unordered	65.75 $\pm$ 7.35 ^a	31.30 $\pm$ 2.20 ^b	64.95 $\pm$ 6.95 ^a	64.60 $\pm$ 9.00 ^a
BLLEG				
pH	3	5	7	9
α-helix	16.65 $\pm$ 0.15 ^a	3.80 $\pm$ 1.00 ^b	15.60 $\pm$ 0.20 ^a	16.85 $\pm$ 0.25 ^a
β-sheet	7.15 $\pm$ 1.65 ^b	37.20 $\pm$ 2.90 ^a	6.80 $\pm$ 1.90 ^b	3.70 $\pm$ 0.70 ^c
Turns	10.70 $\pm$ 2.10 ^b	18.65 $\pm$ 0.15 ^a	9.80 $\pm$ 2.70 ^b	9.95 $\pm$ 2.35 ^b
Unordered	65.50 $\pm$ 3.60 ^c	40.35 $\pm$ 2.05 ^d	67.80 $\pm$ 4.80 ^b	69.45 $\pm$ 2.85 ^a

The data are expressed as mean values (n = 5) $\pm$ standard deviation. For each row, different letters indicate significant differences (p < 0.05) as determined by ANOVA and Duncan multiple range test.

6.3.16 Emulsion formation and stability

The emulsifying capability of white and blue lupin vicilin and legumin fractions were evaluated at different pH values (3, 5, 7, and 9) and protein concentrations (10, 15, and 20 mg/mL) by measuring the mean oil droplet size ($d_{4,3}$) and emulsion stability (Figure 6.4A). At pH 7, all protein fractions demonstrated excellent emulsifying properties, with small oil droplet sizes ($\sim 7\text{-}8\ \mu\text{m}$) and high emulsion stability (100%), regardless of the protein concentration. This good emulsifying performance can be attributed to the optimal balance between protein solubility and surface hydrophobicity observed in all fractions at pH 7, which facilitates rapid adsorption at the oil-water interface and the subsequent formation of a cohesive interfacial film (Ma et al., 2024). No significant differences were observed among protein fractions or between lupin species at this pH, suggesting that the vicilin and legumin proteins from both lupin species possess comparable interfacial properties under neutral conditions where their native structural conformations are maintained, as confirmed by the CD spectroscopy showing higher alpha helix content. At pH 3, all the fractions also maintained relatively small oil droplet sizes ($7\text{-}8\ \mu\text{m}$) and excellent emulsion stability (95-100%), indicating robust emulsification performance despite the acidic environment. This finding is particularly notable for food applications that require acidic conditions, such as fruit-based protein beverages or acidified dairy alternatives. The emulsifying capacity at acidic pH correlated well with the moderate solubility and surface hydrophobicity exhibited by all fractions at pH 3, which enabled effective protein adsorption at the oil-water interface despite the partial conformational changes observed in the CD spectroscopy data. Similar emulsification behavior has been reported for Bambara (Yang et al., 2022) and faba bean (Liu et al., 2022) protein fractions, although lupin proteins appear to maintain superior emulsion stability at acidic pH compared to these proteins. The most pronounced differences in emulsifying properties were observed at pH 5. Blue lupin vicilin significantly outperformed all other fractions, maintaining good emulsifying

properties with relatively small droplet sizes (8-9 μm) and high emulsion stability (95-100%), whereas the other fractions, especially blue lupin legumin, had large oil droplet size (14-18 μm). Protein concentration had a positive influence on stability, with 20 mg/mL consistently outperforming the lower concentrations at pH 5 for BLLEG and WLLEG. The superior performance of BLVL at pH 5 is interesting considering the minimal protein solubility observed at this pH, suggesting that the emulsifying capability of BLVL near the isoelectric point relies primarily on its high surface hydrophobicity rather than solubility, allowing for strong anchoring at the oil-water interface even in the aggregated protein form (Östbring et al., 2021; Tian et al., 2021). At pH 9, all fractions formed emulsions with small droplet sizes ranging from 4-6 μm , which were significantly smaller than those at other pH values, with the legumin fractions producing particularly fine emulsions (4-5 μm). This enhanced oil droplet size reduction at alkaline pH correlates with the increased negative charge (zeta potential -20 to -27 mV) and high solubility of all fractions at pH 9, allowing for rapid diffusion to the interface and efficient steric stabilization. The concentration dependence was most pronounced at this pH, with higher protein concentrations (20 mg/mL) generally producing smaller droplets and more stable emulsions, consistent with the increased availability of emulsifiers at the oil-water interface as described for flaxseed protein fractions (Nwachukwu & Aluko, (2018). Overall, the change in pH had a more significant influence on emulsion formation compared to protein concentration, underscoring the critical role of environmental modification in different food systems. This is because the oil droplet size averaged 8.2 ± 2.8 to 8.6 ± 3.2 μm based on protein concentrations which is significantly ($p < 0.05$) narrower (0.4 μm) than the 6.1 μm (6.0 ± 1.6 to 12.1 ± 3.0 μm range) for the pH values.

6.3.17 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) enabled visualization of the emulsion microstructure, revealing the spatial arrangement of protein (green fluorescence) and oil (red/orange fluorescence) phases in lupin protein-stabilized emulsions at different pH values (Figure 6.4B). These micrographs offer direct visual evidence that complements the quantitative emulsion oil droplet size data. At pH 3, all lupin protein fractions produced emulsions with relatively small oil droplets ($<9\ \mu\text{m}$) that were uniformly distributed throughout the continuous phase. Blue lupin vicilin and white lupin legumin emulsions displayed continuous small droplets with visible protein adsorption at the interface, as indicated by the yellow/orange coloration at the droplet boundaries where the protein and oil signals overlapped. Blue lupin vicilin (BLVL) produced fine droplets with extensive protein coverage, which correlates with its strong emulsifying properties at acidic pH values. Blue lupin legumin (BLLEG) produced slightly larger droplets with a less uniform size distribution compared to other fractions, although adequate protein adsorption at the interfaces was still maintained. At pH 5, significant differences in emulsion microstructure were observed. BLVL maintained relatively small and uniformly distributed droplets ($<11\ \mu\text{m}$) with strong protein adsorption, confirming its good emulsification capacity at the isoelectric point. In contrast, WLVL and BLLEG showed substantial coalescence, with larger oil droplets and a less uniform size distribution. BLLEG exhibited the poorest microstructure at pH 5, with several large and coalesced droplets visible, which is consistent with the reduced emulsion stability at this pH. This pattern is similar to a previous work (Keivaninahr et al., 2021), which reported the presence of irregular and extensive oil phases, indicating emulsion destabilization under these conditions. At pH 7 and 9, all protein fractions produced emulsions with a fine droplet structure and excellent protein coverage at the interfaces, as evidenced by the predominant green fluorescence surrounding distinct red oil droplets. The emulsions at these pH

values exhibited similar microstructural features across all fractions, with WLLEG producing particularly uniform and fine droplets at pH 9. These microstructural characteristics at alkaline pH are similar to those reported by Asen and Aluko (2023) for yellow field pea protein-stabilized emulsions, which exhibited a uniform droplet distribution, indicating efficient protein adsorption at the interfacial layer. The superior emulsifying performance of BLVL, particularly under acidic and alkaline conditions, is clearly reflected in its ability to form and stabilize small oil droplets with effective protein adsorption across these pH values. Compared with CLSM observations by Keivaninahr et al. (2021) for pea protein fractions at acidic pH, BLVL demonstrated more extensive interfacial film formation and better droplet size control.

6.3.18 Foaming properties

Foaming properties are essential functional attributes of proteins in numerous aerated food systems, including whipped toppings, ice creams, meringues, and baked goods. The capacity of proteins to form and stabilize foams depends on their ability to adsorb at air-water interfaces rapidly, undergo partial unfolding, and form cohesive, viscoelastic films that resist coalescence and disproportionation (Othmeni et al., 2024; Zhang et al., 2025). The foaming capacity (FC) and foam stability (FS) of white and blue lupin vicilin and legumin fractions were evaluated at different pH values and protein concentrations (Figure 6.5). Blue lupin vicilin (BLVL) demonstrated superior FC under most pH conditions, reaching high values (75-85%) at pH 3, 5, and 7, particularly at higher protein concentrations (15-20 mg/mL). This FC significantly exceeded that of the other fractions under identical conditions and remained relatively consistent across acidic and neutral pH environments. The foaming performance of BLVL is correlated with its high surface hydrophobicity (459.12) and moderate molecular weight (166 kDa), allowing rapid diffusion and adsorption at the air-water interface. At pH 9, however, all fractions showed modest

and similar FC values (30-45%), suggesting that the high negative charge density interfered with protein-protein interactions necessary to form strong interfacial membranes, regardless of protein type or species origin. The pH dependence of the foaming capacity for BLVL surpasses that reported by Oluwajuyitan and Aluko, (2024) for fava proteins vicilin and legumin, which showed a maximum FC of only 50% at all treated pH conditions. Blue lupin legumin demonstrated the poorest foaming capacity (10-30%) among all fractions, with the lowest foaming capacity observed at neutral pH. The inferior FC of BLLEG fractions compared to BLVL aligns with legumin's larger molecular size and more rigid structure, which limits interfacial flexibility and film formation capacity, consistent with the observations by Shevkani et al. (2015) for kidney bean globulin fractions.

The foam stability patterns differed substantially from the foaming capacity, revealing distinctive mechanisms governing the initial foam formation versus long-term stabilization. The white lupin vicilin demonstrated moderate FS (65-80%) at pH 3 and 9, particularly at 15 mg/mL. This difference between moderate FC and excellent FS suggests that once WLVL adsorbs at the interface, it forms highly cohesive and viscoelastic films that are resistant to drainage and coalescence. This behavior is similar to that observed by Burgos-Díaz et al. (2016) for lupin protein fractions, where the FC and FS typically show parallel trends. While initial foam formation may benefit from high surface hydrophobicity (as seen with BLVL's superior foaming capacity), foam stability depends more on protein-protein interactions. The excellent foam stability of WLVL (65-80%) despite lower surface hydrophobicity can be attributed to its higher disulfide bond content (20.53 $\mu\text{mol/g}$, Table 6.1) and favorable secondary structure characteristics for foam stability at pH 7, with higher α -helix content that contributes to protein flexibility and film elasticity (Table 6.3). Disulfide bonds provide intermolecular cross-linking at the air-water interface, creating

mechanically robust foam films that resist collapse. These disulfide bonds act as covalent stabilizers, preventing foam collapse by maintaining protein network integrity under stress (Zhang et al., 2025). The BLVL showed inconsistent FS despite its excellent FC, with poor stability (30-40%) at pH 3, 5, and 7 but improved stability (40-60%) at pH 9. This pattern suggests that, while BLVL excels at rapid interfacial adsorption and initial foam formation, it lacks the structural characteristics necessary for long-term foam stabilization under most pH conditions (Van de Vondel et al., 2023; Völpl et al., 2021). Blue lupin legumin showed poor FS (10-30%) at most pH values, except pH 5, where it achieved moderate stability (40-50%) at 20 mg/mL. This poor FS suggests that the rigid structure reduces the intermolecular protein interactions, limiting its ability to form a strong viscoelastic film at the interface (Zhang et al., 2025). The better FS of WLVL at pH 3 and 9 makes it particularly suitable for acidic and alkaline aerated foods requiring long-term stability, whereas the superior FC but moderate FS of BLVL suggests its potential application in products where maximum initial aeration is prioritized over extended stability.

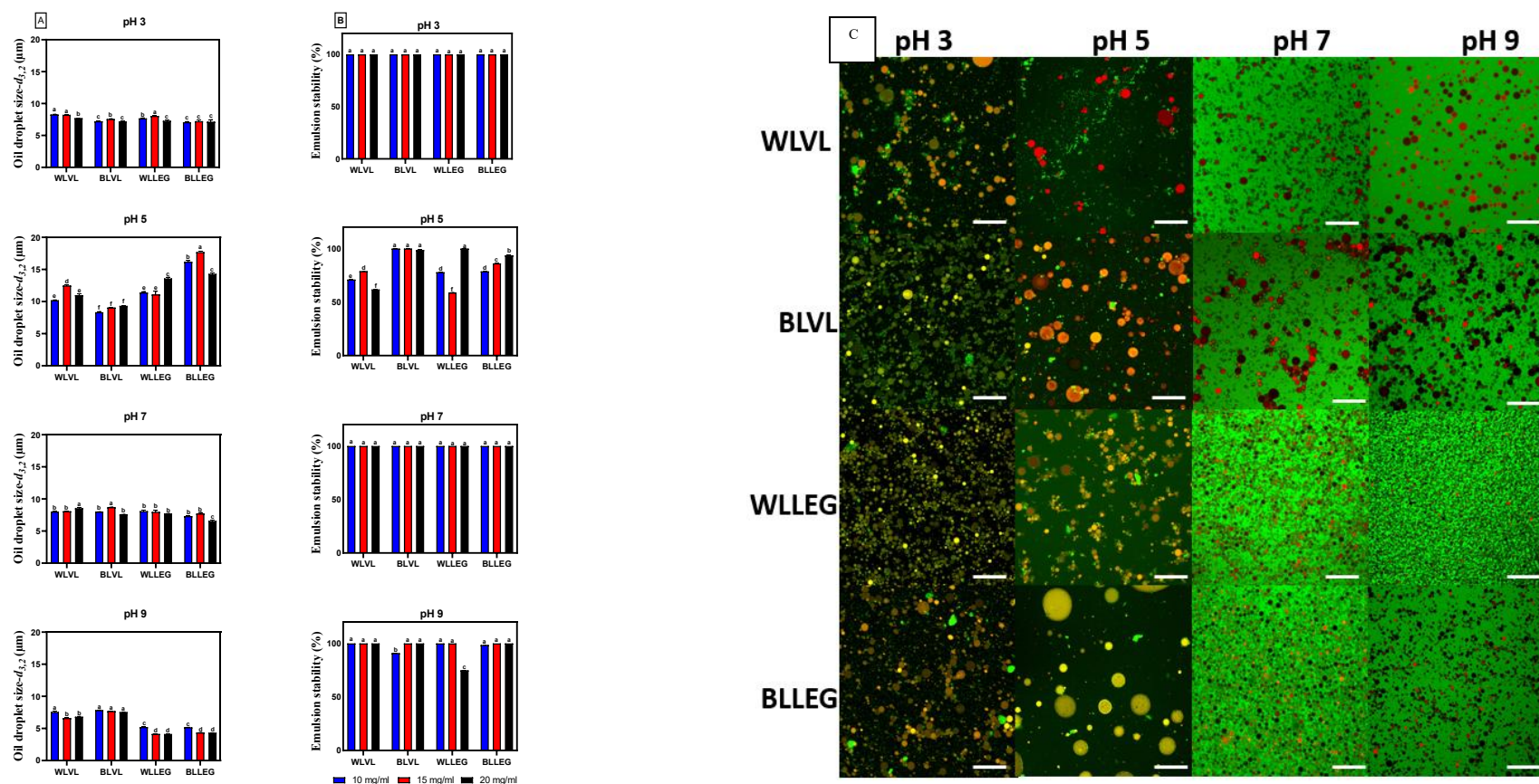


Figure 6.4 (A) Effect of pH and protein concentration on the mean oil droplet sizes ($d_{3,2}$) of blue and white lupin vicilin and legumin fractions. Fig. 4(B). Effect of pH and protein concentration on the emulsion stability of blue and white lupin vicilin and legumin fractions. Fig. 4 (C). Confocal laser scanning microscopy images of freshly prepared emulsions of blue and white lupin vicilin and legumin fractions. BLVL-blue lupin vicilin; WLVL-white lupin vicilin; WLLEG-white lupin legumin; BLLEG-blue lupin legumin. The scale bars within the figures represent 40 μm in length. Values with different superscripts within the same pH are significantly different ($p < 0.05$) as determined by two-way ANOVA and Duncan's multiple range test.

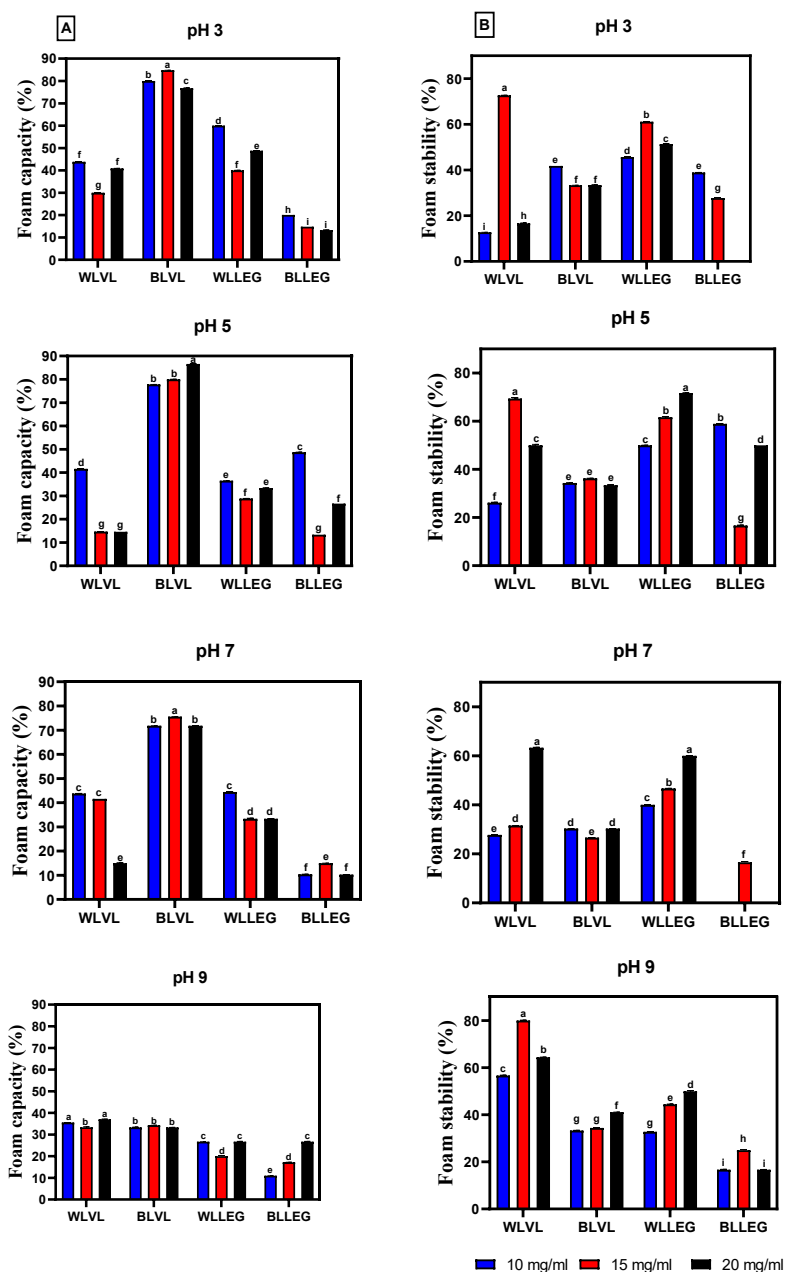


Figure 6.5 (A) Effect of pH and protein concentration on the foaming capacity of blue and white lupin vicilin and legumin fractions. Fig. 5 (B). Effect of pH and protein concentration on the foam stability of blue and white lupin vicilin and legumin fractions. BLVL-blue lupin vicilin; WLVL-white lupin vicilin; WLLEG-white lupin legumin; BLLEG-blue lupin legumin. Values with different superscripts within the same pH are significantly different ($p < 0.05$) as determined by two-way ANOVA and Duncan's multiple range test.

6.4 CONCLUSIONS

The investigation of the structural and functional properties of vicilin and legumin fractions from white and blue lupin seeds revealed distinctive characteristics with significant implications for food applications. Overall, this study concluded that the type of protein (vicilin vs. legumin) and the origin of the lupin species (white versus blue) significantly influence the molecular organization, physicochemical properties, and functionality in food systems. The BLVL emerged as a superior functional ingredient, exhibiting high emulsifying properties across various pH environments, particularly at pH 5, which is suitable for the formation of acidic plant-based beverages. Coupled with its high surface hydrophobicity and molecular flexibility, the BLVL offers significant advantages for applications in complex food systems, such as yogurt, fermented dairy products, and bread dough fermentation, which undergo pH transitions during processing or storage. Similarly, the superior FC of BLVL offers potential advantages for aerated food formulations such as ice cream and whipped cream, where rapid interface adsorption is critical. In addition, WLVL also demonstrated high oil-holding capacity and FS, suggesting its potential applications in fat-reduced meat products and stable foam-based foods. WLLEG exhibited the highest thermal stability, low least gelation concentration, and distinctive secondary structure characteristics, indicating suitability for heat-processed applications requiring protein network formation. The BLLEG showed superior protein recovery and digestibility, suggesting nutritional advantages, as well as good emulsifying capacity and stability at acidic and alkaline pH values. The structure-function relationships observed in this study provide molecular-level explanations for functional property differences. Surface hydrophobicity, molecular size distribution, and pH-dependent conformational changes directly influenced the interfacial behavior and protein-protein interactions in these protein fractions. The distinctive sulfhydryl and disulfide profiles among the fractions further explain their thermal behavior and gelation properties. These findings expand the

current understanding of lupin proteins by revealing that species-specific differences can be as crucial as protein-type classifications. This knowledge enables more targeted selection of specific lupin protein fractions for particular food applications, potentially enhancing product quality while utilizing sustainable plant protein sources. A limitation of this study is the *in vitro* protein digestibility assay (Hsu et al., 1977) because it does not account for *in vivo* factors such as gut microbiota interactions, gastric emptying rates, and individual physiological variations, which may affect actual bioavailability. Additionally, emulsion stability was evaluated over 30 minutes, which, while useful for initial characterization, does not reflect the long-term storage stability required for commercial food applications. Therefore, future studies should incorporate *in vivo* digestibility models and extended stability testing under various storage conditions (temperature, pH, ionic strength) to better assess the commercial viability of these protein fractions for food industry applications. Furthermore, implementing phenolic removal strategies, such as polyvinylpolypyrrolidone treatment or optimized washing protocols with chaotropic compounds or mild detergents, could reduce the co-extraction of phenolic compounds, which may interfere with gelation performance. Additionally, investigating enzyme-assisted extraction protocols using cellulases or pectinases may improve protein release from cell wall matrices and enhance overall extraction efficiency. Furthermore, developing pilot-scale fractionation processes with systematic optimization of processing parameters, including extraction time, dialysis, and centrifugation conditions, would be essential for enhancing both yield and purity for potential industrial applications.

CRedit AUTHORSHIP CONTRIBUTION STATEMENT

Stanley Chukwuejim: Writing – original draft, Investigation, Methodology, Formal analysis.

Deepak Kadam: Methodology, Formal analysis, Writing – review & editing.

Rotimi Emmanuel Aluko: Writing – review & editing, Validation, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

DECLARATION OF COMPETING INTEREST

The author is an Editorial Board Member/Editor-in-Chief/Associate Editor/Guest Editor for Journal of Functional Foods, Food Chemistry, and Food Chemistry X and was not involved in the editorial review or the decision to publish this article. S.C. and D.K. declare no conflict of interest.

DATA AVAILABILITY

Data will be made available on request.

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TRANSITION STATEMENT

In chapter 6 (manuscript 5), the characterization of lupin proteins at the most detailed level of fractionation examined in this thesis was completed by separating vicilin (7S) and legumin (11S) fractions from both white and blue lupin. The results demonstrated that protein type and species origin jointly determine molecular organization, physicochemical properties, and functionality. Blue lupin vicilin (BLVL) emerged as a particularly promising functional ingredient, exhibiting high surface hydrophobicity, molecular flexibility, and superior emulsifying and foaming properties across a wide pH range, including acidic conditions relevant to fermented and beverage applications. White lupin legumin (WLLEG), by contrast, displayed the highest thermal stability and the lowest gelation concentration, suggesting suitability for heat-processed foods that require stable protein network formation. Collectively, Chapters 3 through 6 established that native lupin proteins—whether as whole isolates or individual fractions—possess considerable technofunctional potential but also exhibit limitations under certain processing conditions, particularly near the isoelectric point where emulsification and solubility are compromised. One strategy for overcoming these limitations without altering protein source or pH is enzymatic modification through covalent cross-linking. Transglutaminase-catalyzed polymerization can create high-molecular-weight protein networks that may improve interfacial film strength, emulsion stability, and thermal resilience. Having characterized the native structural and functional baseline of lupin proteins in the preceding chapters, Chapter 7 (Manuscript 6) investigates whether transglutaminase treatment can enhance the emulsifying properties of white and blue lupin protein isolates, examining the relationship between enzyme concentration, degree of cross-linking, and the resulting changes in molecular weight distribution, secondary structure, surface charge, solubility, and emulsion characteristics.

CHAPTER SEVEN

MANUSCRIPT SIX

EMULSIFYING PROPERTIES OF TRANSGLUTAMINASE TREATED WHITE AND BLUE LUPIN PROTEIN ISOLATES

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ABSTRACT

This study investigated the effects of microbial transglutaminase treatment (5-25 U/g protein) on the emulsifying properties of white lupin (WLPI) and blue lupin (BLPI) protein isolates at pH 3, 5, 7, and 9. Transglutaminase catalyzed the formation of high-molecular-weight polymers through ϵ -(γ -glutamyl)lysine cross-linking, as confirmed by SDS-PAGE analysis. FTIR spectroscopy revealed concentration-dependent decreases in β -sheet content (10-15% at 20-25 U/g) with concurrent increases in α -helical structures and aggregated β -sheets. Enzymatic modification induced species-specific and pH-dependent changes in solubility and surface charge characteristics. At pH 3, transglutaminase treatment substantially improved emulsifying capacity, reducing droplet sizes from 14 μm to 7-8 μm for WLPI, with enhanced emulsion stability attributed to the formation of robust interfacial protein films. At pH 9, strong electrostatic repulsion (-20 to -30 mV) dominated the stabilization mechanisms, rendering enzymatic modifications less influential. Confocal microscopy and flocculation/coalescence indices confirmed these pH-dependent microstructural changes. These findings demonstrate that the successful application of transglutaminase-modified lupin proteins requires careful optimization of enzyme concentration and pH to balance structural modifications with functional performance in food emulsion systems.

Keywords

Lupin, transglutaminase, emulsion, coalescence, flocculation, protein structure, confocal laser microscopy

7.1 INTRODUCTION

Lupin (*Lupinus spp.*) is an underutilized legume with unique nutritional qualities, containing about 35-45% protein that rivals soybeans, along with agricultural benefits such as nitrogen fixation and adaptability to marginal soils (Chukwuejim et al., 2023). Among commercially important species, white lupin (*L. albus*) and blue lupin (*L. angustifolius*) have gained increasing attention as sustainable protein sources due to their balanced amino acid profiles, low-alkaloid cultivars, and established cultivation in Mediterranean, Europe, and Australian farming systems (Vogelsang-O'Dwyer et al., 2020). The storage proteins in lupin seeds, mainly the 11S legumin-like globulin (α -conglutin) and 7S vicilin-like globulin (β -conglutin), make up about 85% of total seed proteins and influence their functional properties in food applications such as the development of emulsifiers and foaming agents (Berghout et al., 2014; Devkota et al., 2023). The development of legume-based emulsified food products requires proteins with superior interfacial properties to stabilize oil-water interfaces and prevent phase separation during processing and storage (Grasberger et al., 2024).

Emulsification capacity also depends on multiple protein characteristics, such as molecular flexibility, surface hydrophobicity, charge distribution, and the ability to rapidly adsorb at air-water interfaces and form viscoelastic films (Guldiken et al., 2023). Legume proteins often exhibit suboptimal emulsifying properties compared with dairy proteins due to their compact conformation, limited molecular flexibility, and tendency to aggregate near neutral pH (Cabrita et al., 2023). These limitations become particularly pronounced in complex food matrices requiring long-term stability, thermal processing resistance, and compatibility with various ionic strengths (Östbring et al., 2021). The growing demand for clean-label, sustainable food ingredients has intensified interest in enhancing plant protein functionality through targeted modifications rather than relying on synthetic emulsifiers or animal-derived proteins (Nivala et al., 2021).

Recognition of the techno-functional limitations of lupin proteins has prompted extensive investigation into modification strategies to improve their sensory attributes, functionality, and processing behavior. Physical treatments, including high-pressure processing (Lo et al., 2024; Xia et al., 2019), ultrasound application (Lo et al., 2022), supercritical carbon dioxide (Domínguez-Valencia et al., 2025), and controlled thermal treatments (Yaver & Bilgiçli, 2023), have demonstrated moderate success in enhancing solubility and reducing aggregate formation through disruption of non-covalent interactions and conformational changes. Bioprocessing approaches are also another promising alternative, with germination increasing proteolytic activity to generate peptides with improved functionality (Guzmán-Ortiz et al., 2024; Navarro-Vozmediano et al., 2025), while fermentation with selected microorganisms can reduce anti-nutritional factors and modify flavor profiles (Schlegel et al., 2019). Chemical modifications through acetylation (Heredia-Leza et al., 2022), phosphorylation (Zhang et al., 2024), and Maillard-induced glycation (Kutzli et al., 2021) have shown potential for enhancing emulsification properties, though concerns regarding processing severity and regulatory acceptance limit their commercial application. Among enzymatic approaches, proteolytic hydrolysis has been extensively studied, generating peptides with enhanced solubility but often poor sensory profile due to the generation of bitter peptides (Opazo-Navarrete et al., 2022; Pasarin et al., 2023). These varied modification strategies highlight the ongoing challenge of simultaneously improving multiple functional attributes while maintaining protein structural integrity and clean-label status.

Transglutaminase (protein-glutamine γ -glutamyltransferase, EC 2.3.2.13) catalyzes the formation of covalent ϵ -(γ -glutamyl)lysine cross-links between protein molecules, offering a targeted approach for improving protein functionality without extensive hydrolysis or harsh chemical treatments (Nivala et al., 2021). This enzyme's ability to create inter- and intramolecular

isopeptide bonds has been successfully exploited to modify various legume proteins, with studies demonstrating enhanced gel strength, improved emulsion stability, and modified film-forming properties (Huang et al., 2022; Kang et al., 2024; Santoso et al., 2024). In soy proteins, transglutaminase treatment increased the emulsifying activity index through controlled polymerization, enhancing interfacial film strength without compromising protein structural integrity (Huang et al., 2022). Investigations with pea and faba bean proteins revealed that moderate cross-linking optimally balances molecular weight increases with improved surface activity, whereas excessive treatment leads to reduced functionality due to the formation of extensively cross-linked aggregates (Nivala et al., 2021; Yaputri et al., 2023). The enzyme's specificity for glutamine and lysine residues, which are abundant in legume storage proteins, suggests particular suitability for lupin protein modification. Transglutaminase-induced cross-linking can enhance emulsification through multiple pathways, such as increasing molecular weight to improve steric stabilization, creating amphiphilic structures through the selective cross-linking of hydrophobic and hydrophilic domains, and strengthening interfacial films through the formation of a covalent network (Zhan et al., 2022).

Despite growing interest in enzyme-modified plant proteins, critical knowledge gaps exist regarding transglutaminase treatment of lupin proteins from different lupin species. The relationship between the cross-linking degree and emulsifying properties of lupin proteins remains poorly understood, with no systematic investigation of enzyme concentration effects on interfacial behavior, droplet stabilization mechanisms, and emulsion microstructure. Furthermore, the molecular basis for any improvements, including changes in protein conformation, surface characteristics, and aggregation behavior, needs to be elucidated to enable the rational optimization of treatment conditions. The comparative efficacy of transglutaminase treatment between white

and blue lupin proteins, which differ in their amino acid profiles and structural organization, has not been explored, despite potential implications for commercial applications.

The novelty of the present work lies in: (1) the first systematic comparison of transglutaminase modification effects on emulsifying properties between white and blue lupin protein isolates; (2) comprehensive evaluation across a wide pH range (pH 3, 5, 7, and 9), revealing distinct pH-dependent responses that have practical implications for food formulation; (3) integration of multiple analytical approaches (SDS-PAGE, FTIR, zeta potential, CLSM, flocculation/coalescence indices) to establish structure-function relationships between cross-linking extent, secondary structure changes, and emulsifying performance; and (4) identification of optimal modification conditions (10-15 U/g enzyme concentration) that balance cross-linking benefits with maintained solubility and functionality. These findings address a critical research gap by demonstrating how transglutaminase modification can be strategically applied to lupin proteins for targeted food applications, particularly in acidic emulsified systems.

We hypothesize that the species-specific differences in amino acid profile and changes in transglutaminase enzyme concentration will result in distinct structural and functional properties, highlighting the role of species selection in the development of tailored functional ingredients. Therefore, the objective of this study was to investigate the effect of transglutaminase treatment on the emulsifying properties of protein isolates from white and blue lupin seeds. The outcome is expected to establish a structure-function relationship between transglutaminase-mediated enhancement of lupin protein and its emulsifying properties, which will contribute to the development of improved plant-based food ingredients.

7.2 MATERIALS AND METHODS

7.2.1 Materials

The seeds of white lupin (*Lupinus albus* 'Dieta') and blue lupin (*Lupinus angustifolius* 'Boregine') were provided by The Lupin Platform Inc. (Calgary, Alberta, Canada). Microbial transglutaminase (EC 2.3.2.13) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Pure Valley 100% canola oil was used for emulsion preparation. Sodium dodecyl sulfate (SDS), β -mercaptoethanol, Coomassie Brilliant Blue R-250, 8-anilino-1-naphthalenesulfonic acid (ANS), Tris-HCl, and sodium chloride were also obtained from Sigma-Aldrich (St. Louis, MO, USA). Precision Plus Protein Dual Xtra Standards were purchased from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals and reagents utilized in this investigation were of analytical grade purity and procured from either Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific Company (Oakville, ON, Canada). Ultrapure water was obtained from a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA) and employed for all solution preparations.

7.2.2 Methods

7.2.2.1 Experimental design

The experimental design was structured to evaluate the effects of protein source, enzymatic modification, and pH on the structural, interfacial, and emulsifying properties of lupin protein isolates. Two protein types (white lupin protein isolate, WLPI, and blue lupin protein isolate, BLPI) were investigated. Transglutaminase concentration (5, 10, 15, 20, and 25 U/g protein) and pH (3, 5, 7, and 9) were considered as experimental variables. Protein solubility was initially assessed over a broader pH range to characterize overall pH-dependent behavior (pH 3-9), while detailed structural (zeta potential, SDS-PAGE, FTIR) and functional (emulsification, microstructure, flocculation, and coalescence) analyses were conducted at the selected pH values. The measured response variables included solubility, surface charge, molecular weight

distribution, secondary structure composition, droplet size, emulsion microstructure, and stability indices.

7.2.2.2 Sample preparation

Lupin protein isolates were prepared by isoelectric pH precipitation as previously described (Chukwuejim & Aluko, 2025). Microbial transglutaminase (mTG) was dissolved in 20 mM Tris-HCl buffer containing 150 mM NaCl (pH 8.0) to prepare the enzyme stock solution. The enzymatic polymerization was carried out according to a previous method (Nivala et al., 2021), which was slightly modified as follows. Lupin protein isolates were treated with varying enzyme concentrations: 5, 10, 15, 20, and 25 U/g protein. Enzymatic cross-linking was conducted by incubating protein-enzyme mixtures at 40 °C for 2 h with gentle agitation. Following incubation, enzyme activity was terminated by heat treatment at 95°C for 5 min. The mixture was cooled with ice, and freeze-dried to obtain a dry powder, which was stored at -20 °C. Control samples underwent identical processing conditions, but without enzyme addition.

7.2.2.3 Determination of free amino groups

The content of free primary amino groups in the control and transglutaminase treated protein isolates was determined using the o-phthalaldehyde (OPA) assay, with minor modifications (Boachie et al., 2022). In brief, borate buffer was prepared by dissolving 3.81 g of sodium tetraborate in 80 mL of Milli-Q water. This was followed by the addition of 0.088g dithiothreitol and 0.1g sodium dodecyl sulphate. The OPA reagent was freshly prepared by dissolving 80 mg of OPA in 2 mL of ethanol. The freshly prepared OPA was added to the borate buffer and was made up to 100 mL with Milli-Q water. The reagent was mixed and protected from light until use. Control and TGase-treated protein samples were diluted with Milli-Q water to the same protein concentration (1 mg/mL) to ensure direct comparison between treatments, and all samples were

equilibrated to room temperature prior to analysis. For the assay, 200 μ L of OPA reagent was mixed with 50 μ L of protein sample in a microplate well, corresponding to an OPA reagent-to-sample ratio of 4:1 (v/v). The reaction mixtures were gently mixed and incubated at room temperature for 2 min, after which absorbance was measured at 340 nm using a microplate reader (Biotek Synergy H4, Vermont, USA). Quantification was performed using a calibration curve prepared with L-serine (0–1.0 mM) dissolved in Milli-Q water, and results were expressed as millimoles of serine equivalents per gram of protein.

7.2.2.4 Zeta potential

Surface charge characterization of transglutaminase polymerized lupin protein isolates was performed according to the procedure described by Chukwuejim et al. (2025). A Zetasizer Nano-Z (Malvern Instruments Ltd., UK) was employed for zeta potential determinations at pH 3, 5, 7, and 9. Protein dispersions (0.1% w/v) were prepared in various buffer systems: acetate buffer (0.1 M, pH 3 and 5), phosphate buffer (pH 7), and Tris-HCl buffer (pH 9). Samples underwent overnight equilibration at 4 °C with continuous stirring. Before measurement, dispersions were equilibrated to ambient temperature and clarified by centrifugation ($2,000 \times g$, 10 min) to eliminate insoluble materials. Zeta potential measurements utilized automatic voltage selection mode, and values were calculated applying the Smoluchowski approximation with instrument parameters set at refractive index 1.45 and absorption coefficient of 0.001.

7.2.2.5 SDS-PAGE

Electrophoretic characterization of transglutaminase polymerized lupin protein isolates was performed using Mini-PROTEAN TGX precast 4-15% gradient gels (Bio-Rad Laboratories, Hercules, CA, USA) under reducing condition, following modified procedures from Laemmli (1970). Protein samples were diluted to 10 mg/mL in sample buffer containing 62.5 mM Tris-HCl

(pH 6.8), 2% (w/v) SDS, 25% (v/v) glycerol, 10% (v/v) mercaptoethanol, and 0.01% (w/v) bromophenol blue. The mixtures were heated at 95 °C for 5 min, cooled and centrifuged. Protein samples (10 μ L per lane) were loaded alongside a molecular weight marker (10-250 kDa). Electrophoresis was conducted at a constant voltage of 150 V for approximately 70 min in Tris-glycine-SDS running buffer (25 mM Tris, 0.192 mM glycine, 0.1% SDS, pH 8.3) until the dye front reached the gel bottom. Following electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 (0.1% in 40% methanol, 10% acetic acid) for 2 h with gentle agitation, then destained in 50% methanol:10% acetic acid:40% DDW solution until a clear background was achieved. Protein bands were visualized and documented using a gel imaging system (UMAX, Dallas, TX, USA).

7.2.2.6 Secondary structure analysis by FTIR

Protein secondary structure characterization was performed using Fourier-transform infrared spectroscopy (Bruker Invenio S, Bruker Optics, Germany) following the method described by Hewage et al. (2024). Approximately 2-3 mg of each transglutaminase polymerized lupin protein isolate was placed directly on the diamond ATR crystal, and uniform contact was ensured using the pressure applicator. Spectral acquisition parameters were set as follows: resolution 4 cm^{-1} , scanning range 4000-400 cm^{-1} , with 120 scans averaged per spectrum. Background spectra were collected for 1 min before each sample measurement using identical parameters and automatically subtracted. The amide I region (1700-1600 cm^{-1}) was analyzed for secondary structure determination. Spectral data were exported and processed using Origin software (OriginLab Corporation, USA). Processing involved baseline correction, smoothing, and calculation of second-order derivatives to resolve overlapping peaks. Peak deconvolution was performed through Gaussian curve fitting using Origin's peak analysis tools. Secondary structure assignments were

based on characteristic absorption bands: β -sheets (1627-1640 cm^{-1} and 1670-1680 cm^{-1}), α -helices (1650-1660 cm^{-1}), β -turns (1680-1690 cm^{-1}), Aggregated Sheets A₁ (1615-1625 cm^{-1}), Aggregated Sheets A₂ (1695-1700 cm^{-1}) and random coils (1640-1650 cm^{-1}). The relative proportion of each structure was calculated from the integrated areas of assigned peaks using Origin software.

7.2.2.7 Protein solubility

Solubility profiles for the transglutaminase polymerized lupin protein isolates were obtained according to the methodology of Chukwuejim & Aluko, (2024). For each condition (pH 3-9), 50 mg transglutaminase polymerized protein isolate (or control) was dispersed in 5 mL of the appropriate buffer. Samples were vortexed thoroughly for 30 s and equilibrated for 60 min at room temperature to ensure complete hydration. Insoluble material was removed by centrifugation (5,600 $\times$ g, 30 min, 25 $^{\circ}\text{C}$), and the supernatant analyzed for protein content using the modified Lowry assay (Markwell et al., 1978). To establish total protein content, parallel samples were also solubilized in 1 M NaOH. Protein solubility (PS, %) was calculated as:

$$PS (\%) = \frac{\text{protein content of the supernatant at a specific pH}}{\text{total protein content of the sample}} \times 100 \quad (1)$$

7.2.2.8 Emulsification ability and emulsion stability

Oil-in-water emulsions were formulated following a modified protocol based on Asen & Aluko, (2023). Protein dispersions (10 mg/mL, based on protein content) were prepared in different 0.1 M buffer systems to achieve varying pH conditions: acetate (pH 3), phosphate (pH 5 and 7), and Tris (pH 9). To form emulsions, 1 mL of refined canola oil (Pure Valley 100% canola oil, Montreal, QC) was combined with 5 mL of each protein dispersion. Homogenization was performed at 20,000 rpm for 60 s using a Polytron PT 10-35 homogenizer (Kinematica AG, Lucerne, Switzerland) fitted with a 12-mm dispersing aggregate to achieve uniform droplet dispersion.

Particle size distribution was characterized via laser diffraction using a Mastersizer 3000 (Malvern Panalytical Ltd., Malvern, UK). Measurements were performed using the Hydro 3000 small-volume dispersion unit with Milli-Q water as the continuous phase. Emulsion samples were introduced into approximately 100 mL of water under continuous stirring until optimal obscuration was achieved. The surface-weighted mean diameter ($d_{3,2}$) was determined from triplicate measurements and used as an index of emulsification ability. Short-term emulsion stability was evaluated by comparing initial droplet sizes with those measured after 30 min of undisturbed storage at room temperature. It should be noted that this measurement reflects short-term physical stability rather than long-term shelf stability. The short-term emulsion stability was calculated using the formula;

$$\text{Emulsion stability (\%)} = \frac{\text{Mean oil droplet size at 0 min}}{\text{Mean oil droplet size at 30 min}} \times 100 \quad (2)$$

A value of 100% indicates no change in droplet size, reflecting complete short-term stability. Values below 100% indicate an increase in droplet size over the 30 min period, suggesting destabilization through coalescence or flocculation. Values exceeding 100% may occasionally occur due to measurement variability or minor redistribution effects and should not be interpreted as improved stability

7.2.2.9 Microstructure characterization of emulsions

Confocal laser scanning microscopy (CLSM) was used to analyze emulsion microstructure following a modified protocol from Tian et al. (2020). Imaging was performed using a Zeiss LSM 700 confocal system equipped with a Zeiss Imager M2 microscope (Carl Zeiss, Oberkochen, Germany). Dual-fluorescence labeling was achieved by staining samples with Nile Red (1 mg/mL in ethanol) for the lipid phase, while fluorescein isothiocyanate (FITC, 1 mg/mL in ethanol)

labeled the protein components. Fluorescence detection was carried out at excitation/emission wavelengths of 561 nm for Nile Red-stained oil droplets and 488 nm for FITC-labeled proteins.

7.2.2.10 Coalescence and flocculation indices

The flocculation index (FI) was determined on freshly prepared emulsions using established protocols (Chukwuejim & Aluko, 2024). Fresh emulsion samples were diluted using two different media: distilled deionized water (DDW) and a 10 mg/mL sodium dodecyl sulfate (SDS) solution. The volume-weighted mean diameter ($d_{4,3}$) was measured for the dispersed oil droplets at two time points: immediately after preparation (0 h) and after 24 h of storage. Particle size analysis was performed using the Mastersizer 3000 (Malvern Panalytical Ltd., Malvern, UK). The FI (%) was calculated using the following equation:

$$FI (\%) = [(d_{4,3} \text{ in water}) / (d_{4,3} \text{ in 10 mg/mL SDS}) - 1] * 100 \quad (3)$$

CI was determined after 24 h of storage at 4 °C as follows:

$$CI (\%) = [(d_{4,3} \text{ 24 h}) / (d_{4,3} \text{ 0 h}) - 1] * 100 \quad (4)$$

where $d_{4,3}$ (0 h) and $d_{4,3}$ (24 h) are the $d_{4,3}$ values of droplets in the emulsion at 0 h and 24 h using 10 mg/mL SDS as the diluent, respectively.

7.2.2.11 Statistical analysis

All experiments were conducted in triplicate, and results are presented as mean $\pm$ standard deviation. Statistical analysis was performed using IBM SPSS statistical software version 28 (Chicago, IL, USA). For datasets in which WLPI and BLPI were analyzed in separate panels (free amino group content, zeta potential, and protein solubility), one-way ANOVA was conducted for each protein isolate at each pH level, with enzyme treatment (0–25 U/g) as the factor. For datasets

in which both protein isolates were compared within the same figure (emulsification capacity, emulsion stability, flocculation index, and coalescence index), a one-way ANOVA was conducted at each pH level, with all protein types and enzyme-treatment combinations as factors. Interactions among protein type, enzyme concentration, and pH were not statistically tested in this study. In all cases, significant differences between means were determined using Duncan's multiple-range test at the $p < 0.05$ significance level.

7.3 RESULTS AND DISCUSSION

7.3.1 Free amino group content

The free amino group content can be linked to the degree of enzyme cross-linking and provides an indication of intensity of the enzyme activity. Generally, the content of free amino groups decreased progressively with increasing transglutaminase (TG) concentration in both WLPI and BLPI, confirming successful cross-linking (Figure 7.1A & B). For WLPI, free amino groups decreased significantly from 27% (untreated) to approximately 14% at 20-25 U/g, representing a 48% reduction (Fig. 7.1A). BLPI showed a similar trend, declining from 29% to approximately 10% at the highest enzyme concentrations, corresponding to 66% reduction (Fig. 7.1B). The decreased levels of free amino groups can be attributed to TG-catalyzed formation of ϵ -(γ -glutamyl)lysine isopeptide bonds between glutamine and lysine residues, which consumes available primary amines. Notably, BLPI exhibited a distinct threshold behavior, showing minimal change from 5-15 U/g followed by a dramatic drop at 20 U/g, suggesting a critical enzyme concentration is needed to overcome initial structural barriers. In contrast, WLPI showed more gradual kinetics, with reduction plateauing beyond 10 U/g, indicating partial saturation of accessible lysine residues constrained by steric hindrance. These differences likely reflect

variations in lysine accessibility, lysine-to-glutamine spatial proximity, and native protein conformation between varieties.

Table 7.1 Effect of pH and enzyme concentration on the protein solubility of transglutaminase polymerized lupin protein isolates.

pH	BLPI	BT 5U	BT 10U	BT 15U	BT 20U	BT 25U
3	60.97 ± 2.41 ^c	67.89 ± 2.06 ^a	64.30 ± 3.79 ^b	52.76 ± 2.53 ^e	55.33 ± 3.54 ^d	68.58 ± 1.07 ^a
4	28.28 ± 0.12 ^a	25.84 ± 0.89 ^b	15.07 ± 0.75 ^c	11.57 ± 0.67 ^d	9.35 ± 0.79 ^e	9.86 ± 0.24 ^e
5	1.74 ± 0.62 ^c	8.15 ± 0.91 ^a	0.46 ± 0.00 ^c	3.79 ± 0.55 ^b	0	0
6	33.62 ± 3.41 ^a	26.35 ± 0.41 ^b	18.92 ± 1.99 ^c	17.38 ± 0.91 ^c	15.93 ± 0.20 ^d	13.53 ± 0.55 ^d
7	64.99 ± 3.02 ^c	73.45 ± 0.67 ^a	62.34 ± 1.67 ^d	69.35 ± 3.14 ^b	62.85 ± 0.32 ^d	68.23 ± 1.39 ^b
8	90.86 ± 0.12 ^b	95.31 ± 0.59 ^a	89.31 ± 3.66 ^b	86.64 ± 0.98 ^c	90.75 ± 0.96 ^b	86.64 ± 1.47 ^c
9	94.55 ± 1.54 ^b	97.05 ± 1.05 ^a	86.52 ± 1.36 ^d	90.77 ± 1.08 ^c	90.94 ± 1.59 ^c	87.51 ± 1.02 ^d
pH	WLPI	WT 5U	WT 10U	WT 15U	WT 20U	WT 25U
3	44.99 ± 2.61 ^c	51.74 ± 0.55 ^a	45.76 ± 0.31 ^c	43.11 ± 0.12 ^d	47.55 ± 1.98 ^b	45.24 ± 0.84 ^c
4	10.71 ± 0.20 ^b	16.01 ± 0.67 ^a	8.66 ± 0.20 ^c	10.20 ± 0.2 ^b	9.94 ± 0.20 ^{bc}	8.92 ± 0.55 ^c
5	4.05 ± 0.51 ^b	6.27 ± 1.67 ^a	5.76 ± 1.69 ^{ab}	4.22 ± 0.73 ^b	2.07 ± 0.72 ^c	2.51 ± 0.20 ^c
6	44.30 ± 4.20 ^a	44.13 ± 1.39 ^a	43.11 ± 0.24 ^a	36.61 ± 0.36 ^c	40.63 ± 0.31 ^b	38.75 ± 2.14 ^c
7	55.58 ± 2.72 ^a	52.42 ± 1.58 ^b	51.05 ± 1.15 ^c	47.29 ± 0.24 ^d	55.07 ± 0.20 ^a	52.94 ± 0.79 ^b
8	90.64 ± 0.49 ^b	93.08 ± 3.83 ^a	85.53 ± 0.54 ^c	83.11 ± 2.20 ^d	89.97 ± 1.57 ^b	88.64 ± 0.87 ^c
9	91.21 ± 0.59 ^a	91.68 ± 1.31 ^a	89.76 ± 1.92 ^b	82.23 ± 0.68 ^c	91.23 ± 1.32 ^a	88.31 ± 1.52 ^b

Values represent mean ± standard deviation. Values with different superscripts within the same row are significantly different ($p < 0.05$) as determined by ANOVA and Duncan's multiple range test. WLPI-white lupin protein isolate; WT5U-white transglutaminase 5 units; WT10U-white transglutaminase 10 units; WT15U-white transglutaminase 15 units; WT20U-white transglutaminase 20 units; WT25U-white transglutaminase 25 units; BLPI-blue lupin protein isolate; BT5U-Blue transglutaminase 5 units; BT10U-Blue transglutaminase 10 units; BT15U-blue transglutaminase 15 units; BT20U-blue transglutaminase 20 units; BT25U-blue transglutaminase 25 units.

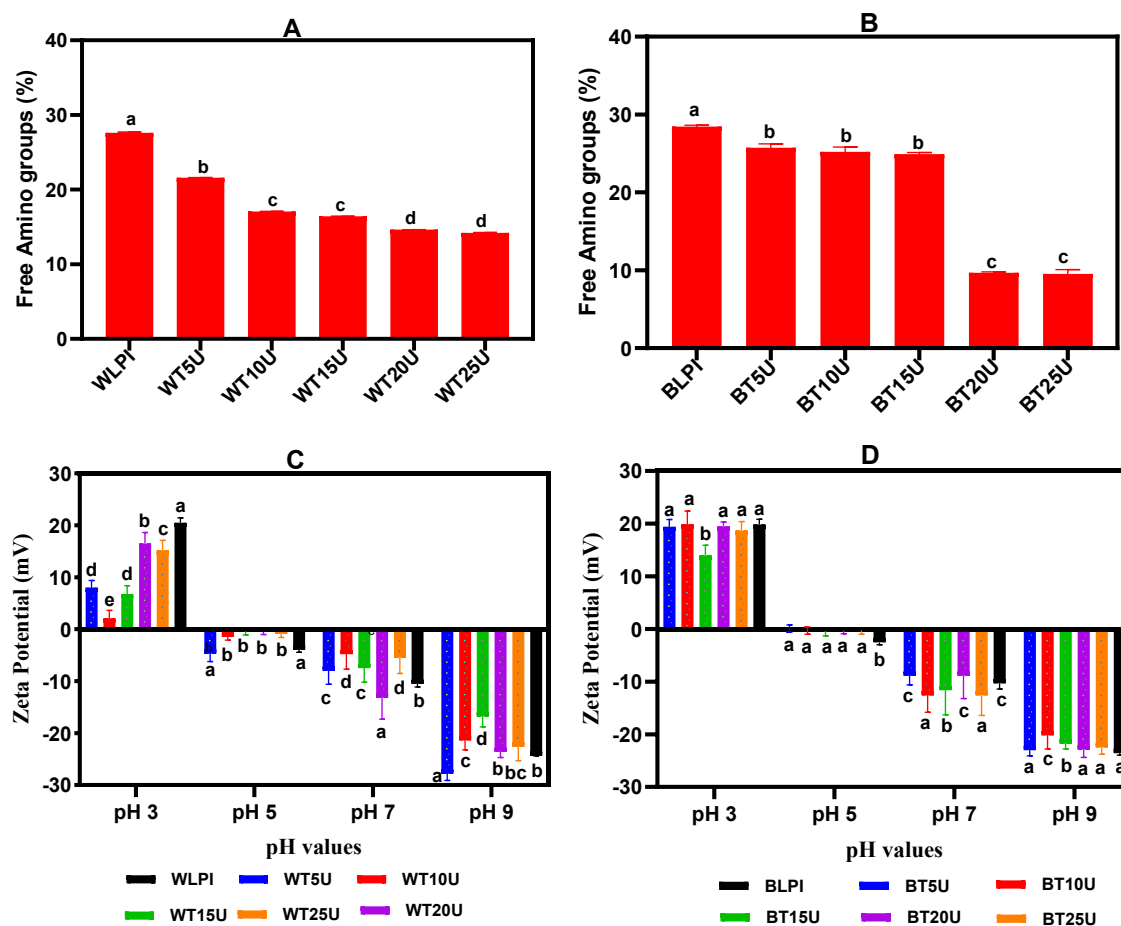


Figure 7.1 Free amino group content of transglutaminase polymerized white (A) and blue (B) lupin protein isolates. At each pH, bars with different letters have significantly different ($p < 0.05$) mean values as determined by ANOVA and Duncan's multiple range test.

Effect of pH and enzyme concentration on the Zeta potential of transglutaminase polymerized white (C) and blue (D) lupin protein isolates. Bars with different letters have significantly different ($p < 0.05$) mean values as determined by ANOVA and Duncan's multiple range test.

WLPI-white lupin protein isolate; WT5U-white transglutaminase 5 units; WT10U-white transglutaminase 10 units; WT15U-white transglutaminase 15 units; WT20U-white transglutaminase 20 units; WT25U-white transglutaminase 25 units; BLPI-blue lupin protein isolate; BT5U-Blue transglutaminase 5 units; BT10U-Blue transglutaminase 10 units; BT15U-blue transglutaminase 15 units; BT20U-blue transglutaminase 20 units; BT25U-blue transglutaminase 25 units.

7.3.2 Zeta potential

The zeta potential profiles displayed characteristic charge reversal patterns for both treated lupin protein isolates, transitioning from positive values at pH 3 (+3 to +20 mV) to strongly negative values at pH 9 (-15 to -30 mV), with the isoelectric point occurring between pH 4-5, where values approached zero (Figure 7.1C & D). This amphoteric behavior reflects the ionization states of constituent amino acids, with histidine, lysine, and arginine residues protonated at acidic pH, while aspartic and glutamic acid residues become deprotonated at alkaline conditions (Nogueira et al., 2019). The comparable magnitude of surface charge between untreated BLPI and WLPI suggests similar surface amino acid compositions despite potential differences in their internal structures (Chukwuejim et al., 2025). TG treatment induced significant modifications to surface charge characteristics, with the most pronounced effects observed at extreme pH values rather than near the isoelectric point. At pH 3, WLPI enzyme-treated samples exhibited similar and slightly enhanced positive charges compared to controls, with values ranging from +3 to +20 mV across all enzyme concentrations. This preservation or even enhancement of the positive charge, despite covalent modification, suggests that TG-mediated cross-linking may expose additional charged groups through conformational changes. Alternatively, the cross-linked network creates a more extended structure with greater surface accessibility of basic residues (Vijayan et al., 2024). The formation of isopeptide bonds consumes lysine residues; yet the maintained positive charge may indicate that either cross-linking predominantly involves buried lysine residues that do not contribute to surface charge, or that protein unfolding during enzymatic treatment exposes previously hidden basic amino acids, compensating for those lost to cross-linking (Nogueira et al., 2019). At alkaline pH, the negative zeta potential values showed minimal variation with enzyme treatment, maintaining values between -15 to -30 mV across all modification levels. This consistency indicates that the acidic residues responsible for negative charge at pH 9 remain

largely unaffected by TG, which specifically targets glutamine and lysine residues rather than acidic amino acids (Nivala et al., 2021). The negative charge at alkaline pH has important implications for emulsion stability, as sufficient electrostatic repulsion can prevent droplet aggregation in cross-linked protein systems (Kang et al., 2024). The zeta potential was observed to be close to zero at pH 5, regardless of enzyme concentration, despite the solubility changes observed at this pH (Table 7.1). This suggests that while extensive cross-linking creates large, insoluble aggregates, the surface charge characteristics of these structures remain similar to native proteins (Nivala et al., 2021). The zeta potential results after TG modification show that treated proteins maintain their polyelectrolyte nature, which is crucial for pH-dependent functionality in food systems. The negative charges observed at pH 7-9 suggest that TG-modified lupin proteins could provide electrostatic stabilization in neutral to alkaline emulsions, while the positive charges at pH 3 suggest suitability for application in acidic food systems.

7.3.3 Gel electrophoresis

The electrophoretic profiles revealed progressive formation of high-molecular-weight polymers with increasing TG concentration, demonstrating the successful enzymatic cross-linking of both white and blue lupin protein isolates (Figures 7.2A and 2B). Native proteins (WLPI and BLPI, lanes G) displayed characteristic band patterns between 15-75 kDa, representing α -conglutin (legumin-like) subunits at approximately 50-60 kDa and β -conglutin (vicilin-like) components at 20-45 kDa, consistent with previous characterization of lupin storage proteins (Duranti et al., 2008). TG treatment induced dose-dependent polymerization, as evidenced by a decrease in the intensity of native molecular weight bands and the concurrent appearance of proteins retained at the gel interface or migrating as diffuse bands above 250 kDa. These high-molecular-weight (>250 kDa) aggregates correlate directly with the substantial reductions in free amino groups at higher

TG concentrations as shown in Fig. 7.1A & B. At moderate enzyme concentrations (5-10 U/g), partial retention of the original band patterns, alongside the emergence of high molecular weight species, suggests incomplete cross-linking, where monomeric and oligomeric forms coexist with newly formed polymers. This heterogeneous modification pattern suggests that TG exhibits preferential reactivity toward specific protein fractions, likely influenced by the accessibility and spatial orientation of glutamine and lysine residues within different protein conformations (San et al., 2025). The extensive polymer formation at 20-25 U/g for WLPI, where substantial protein material remained at the stacking-resolving gel interface, indicates the formation of complexes exceeding 250 kDa that could not penetrate the polyacrylamide matrix. This extensive cross-linking corresponds with the complete loss of solubility observed at pH 5 (Table 7.1), confirming that excessive polymerization creates networks too large to remain in solution. The progressive disappearance of bands in the 20-50 kDa range with increasing enzyme concentration suggests that both acidic and basic polypeptides of the storage proteins participate in cross-linking reactions. The preferential modification of intermediate molecular weight components (30-50 kDa) before smaller peptides (15-25 kDa) implies that larger subunits possess more accessible reactive residues or favorable conformations for enzymatic attack (Nivala et al., 2021). This selective reactivity pattern has important implications for functionality, as complete cross-linking of specific subunits could eliminate their individual contributions to emulsification while creating new polymeric structures with distinct interfacial properties (Vijayan et al., 2024). The coexistence of unmodified proteins, moderate-sized oligomers, and extensive polymers at intermediate enzyme concentrations (10-15 U/g) may offer synergistic effects: smaller proteins rapidly adsorbing at interfaces, intermediate oligomers providing steric stabilization, and large polymers forming thick viscoelastic layers (Zhan et al., 2022).

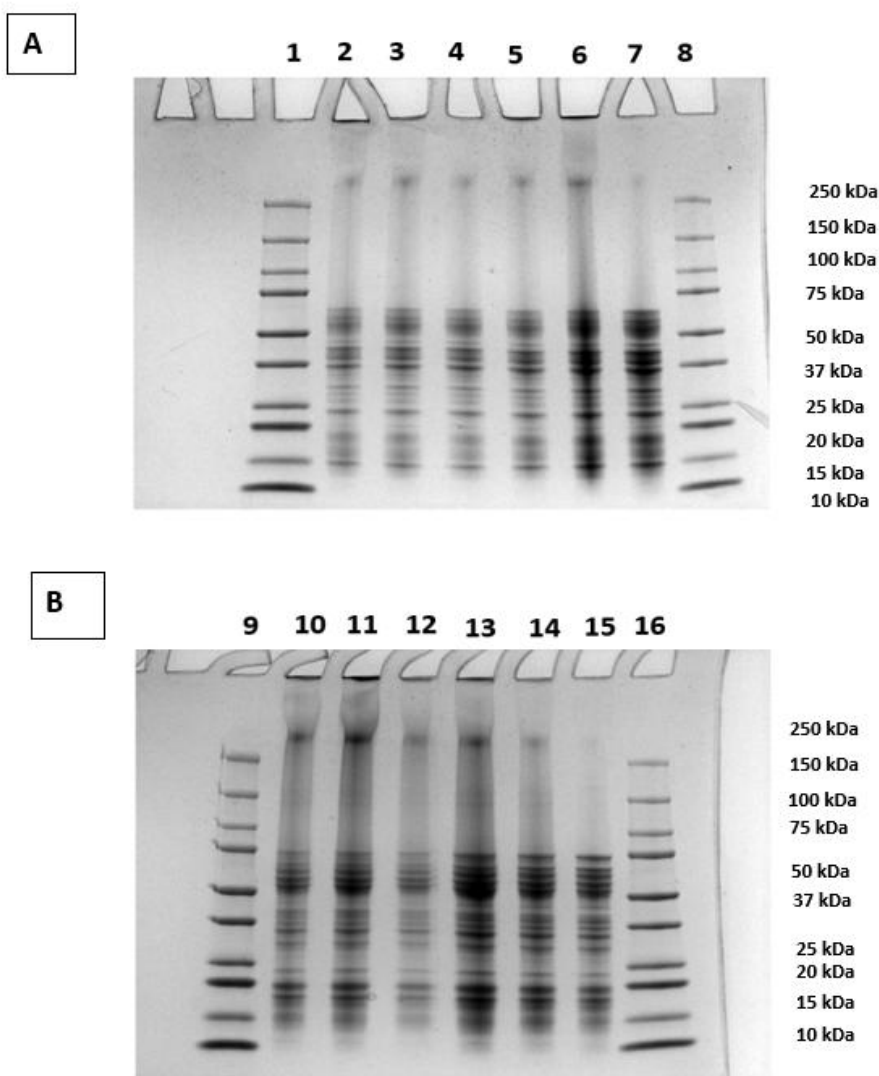


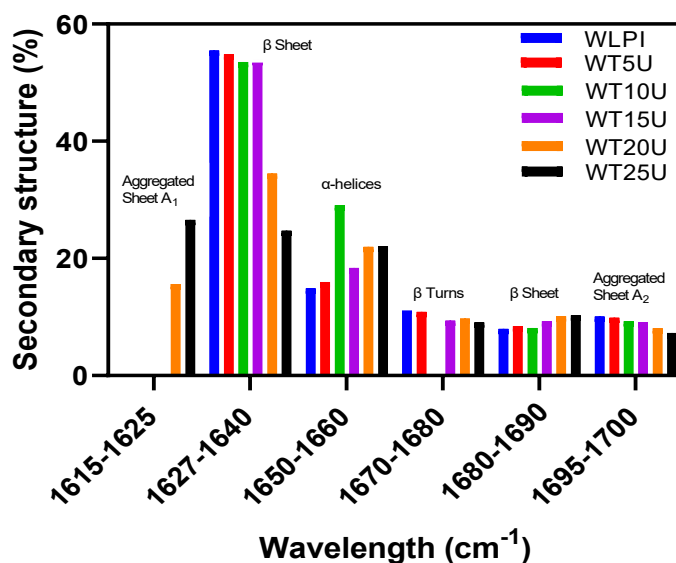
Figure 7.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of transglutaminase polymerized white (A) and blue (B) lupin protein isolates. 1. Protein standard marker (kDa); 2. WT25U; 3. WT20U; 4. WT15U; 5. WT10U; 6. WT5U; 7. WLPI; 8. Protein standard marker (kDa). 9. Protein standard marker (kDa); 10. BT25U; 11. BT20U; 12. BT15U; 13. BT10U; 14. BT5U; 15. BLPI; 16. Protein standard marker (kDa). WLPI-white lupin protein isolate; WT5U-white transglutaminase 5 units; WT10U-white transglutaminase 10 units; WT15U-white transglutaminase 15 units; WT20U-white transglutaminase 20 units; WT25U-white transglutaminase 25 units; BLPI-blue lupin protein isolate; BT5U-Blue transglutaminase 5 units; BT10U-Blue transglutaminase 10 units; BT15U-blue transglutaminase 15 units; BT20U-blue transglutaminase 20 units; BT25U-blue transglutaminase 25 units.

7.3.4 Secondary structure analysis

Analysis of the amide I band ($1700\text{-}1600\text{ cm}^{-1}$) through FTIR spectroscopy allows non-destructive quantification of protein secondary structures because C=O stretching vibrations are sensitive to hydrogen bonding patterns within different conformational states (Tintor et al., 2024). The FTIR spectroscopic analysis revealed that transglutaminase treatment induced alterations in the secondary structure distribution of both lupin protein isolates, with the predominant β -sheet conformation ($1615\text{-}1625$ and $1627\text{-}1640\text{ cm}^{-1}$) comprising approximately 55-60% of total structure in native proteins (Figure 7.3). This β -sheet dominance aligns with the characteristic fold of legume storage proteins, where antiparallel β -sheets form the structural core of both vicilin and legumin proteins (Devkota et al., 2023). The native proteins also displayed substantial α -helical content ($1650\text{-}1660\text{ cm}^{-1}$, approximately 15-20%) and β -turn regions ($1670\text{-}1680\text{ cm}^{-1}$, 10-12%), with minor contributions from random coils ($1640\text{-}1650\text{ cm}^{-1}$) and aggregated β -sheet structures ($1695\text{-}1700\text{ cm}^{-1}$), consistent with FTIR spectroscopic studies of lupin globulins (Devkota et al., 2023). Progressive TG treatment demonstrated a concentration-dependent decrease in β -sheet content, particularly evident at 20-25 U/g where reductions of 10-15% were observed. This structural transformation likely results from the disruption of native β -sheet hydrogen bonding networks during enzymatic cross-linking, as the formation of isopeptide bonds introduces conformational constraints that destabilize regular secondary structures (Wang et al., 2023). The concurrent increase in α -helical content at moderate enzyme concentrations (5-15 U/g) suggests a β -sheet to α -helix transition, potentially induced by local unfolding and refolding events during the cross-linking process (Mattice & Marangoni, 2021). Such structural reorganization has been reported in TG-treated legume proteins, where enzyme-induced treatment facilitates the interconversion of secondary structures (Wang et al., 2023). The emergence of increased aggregated β -sheet structures ($1615\text{-}1625\text{ cm}^{-1}$) at high enzyme concentrations (WT20-25U/g)

corresponds to aggregated β -sheet formation characteristic of protein aggregation. This correlates with the extensive polymerization observed in SDS-PAGE analysis (Fig. 7.2), providing molecular-level evidence for the aggregation observed. Formation of these aggregated β -sheets may also explain the decrease in solubility at pH 4-6, as aggregated β -sheet structures create extended networks stabilized by both covalent cross-links and non-covalent interactions (Devkota et al., 2023). The retention of significant native-like secondary structure at moderate enzyme concentrations (5-15 U/g) has important functional implications. Partial preservation of β -sheet architecture maintains protein stability while introduced cross-links provide additional structural reinforcement, potentially creating proteins with enhanced interfacial properties. The increased α -helical content could improve amphiphilicity, as α -helices often display distinct hydrophobic and hydrophilic faces that facilitate orientation at oil-water interfaces (Mattice & Marangoni, 2021). These structural modifications, combined with increased molecular weight from cross-linking, suggest that moderately modified proteins (10-15 U/g) may offer an optimal balance between structural integrity and functional enhancement for emulsification applications.

A



B

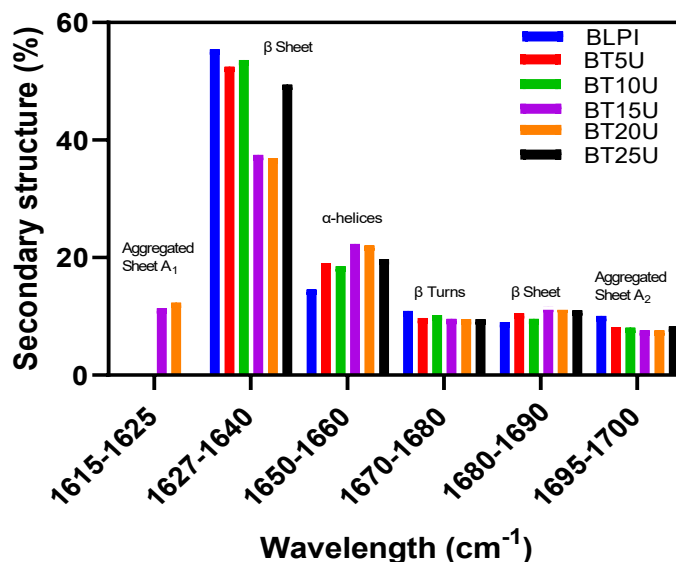


Figure 7.3 Secondary structure analysis from FTIR spectra of transglutaminase polymerized white (A) and blue (B) lupin protein isolates. WLPI-white lupin protein isolate; WT5U-white transglutaminase 5 units; WT10U-white transglutaminase 10 units; WT15U-white transglutaminase 15 units; WT20U-white transglutaminase 20 units; WT25U-white transglutaminase 25 units; BLPI-blue lupin protein isolate; BT5U-Blue transglutaminase 5 units; BT10U-Blue transglutaminase 10 units; BT15U-blue transglutaminase 15 units; BT20U-blue transglutaminase 20 units; BT25U-blue transglutaminase 25 units.

7.3.5 Protein solubility

The pH-dependent solubility profiles of TG-modified lupin protein isolates revealed distinct patterns between the BLPI and WLPI lupin species, with the enzyme concentration significantly influencing the protein solubility behavior across the pH spectrum (Table 7.1). Both treated and untreated proteins exhibited characteristic U-shaped solubility curves with minimal solubility near pH 4-5, corresponding to their isoelectric points where net charge approaches zero and protein-protein interactions predominate over protein-solvent interactions (Opazo-Navarrete et al., 2022). This behavior aligns with previous reports on lupin proteins, which typically exhibit isoelectric points between pH 4-5 due to the predominance of acidic amino acids, such as aspartic acid and glutamic acid, in their primary structure (Chukwuejim & Aluko, 2025). TG treatment induced pronounced solubility reduction at pH 5, with complete insolubilization observed at 20-25 U/g for BLPI, whereas treated WLPI at pH 5 was not completely insoluble. Importantly, this loss of solubility is primarily driven by TG-induced network formation and protein aggregation rather than by surface charge effects alone, as the near-zero zeta potential at the isoelectric point was similar in both treated and untreated samples. This differential response likely reflects structural distinctions between species, particularly the accessibility of glutamine and lysine residues for cross-linking (Nivala et al., 2021). The formation of high molecular weight polymers through ϵ -(γ -glutamyl)lysine bonds reduce protein-water interactions and promotes network formation that entraps water molecules within rigid structures, thereby decreasing apparent solubility (Zhan et al., 2022). The observed effect in BLPI suggests a higher availability of reactive residues or quaternary structural features that favor intermolecular cross-linking. Interestingly, moderate TG treatment (5-10 U/g) maintained or slightly improved solubility at extreme pH values (3 and 8), particularly in the BLPI samples. This phenomenon can be attributed to selective intramolecular cross-linking, which stabilizes the protein conformation against pH-induced unfolding while

preserving the charged residues necessary for electrostatic repulsion (Shen et al., 2022). At acidic and alkaline pH values, the high net charge density overcomes the cohesive forces introduced by cross-linking, maintaining colloidal stability despite increased molecular weight (Zhan et al., 2022). Similar observations with TG-treated soy and pea proteins suggest this represents a general mechanism for enzyme-modified legume proteins (Li et al., 2022; Yang et al., 2023). The progressive decrease in solubility at pH 6-7 with increasing enzyme concentration has implications for food applications, as most emulsified products exist within this range. The retained solubility at pH 3 and 8, however, indicates that pre-solubilization at extreme pH levels before emulsion formation could circumvent the limitations imposed by cross-linking (Li et al., 2022). Furthermore, the partial preservation of solubility at moderate enzyme levels (5-15 U/g) suggests an optimal modification window where enhanced molecular weight and maintained dispersibility coexist, potentially improving emulsifying properties through increased steric stabilization without sacrificing protein availability at the oil-water interface (Mattice & Marangoni, 2021). These solubility patterns establish critical processing parameters for utilizing TG-modified lupin proteins, indicating that enzyme concentration must be carefully controlled to balance structural modification with functional performance in emulsified systems.

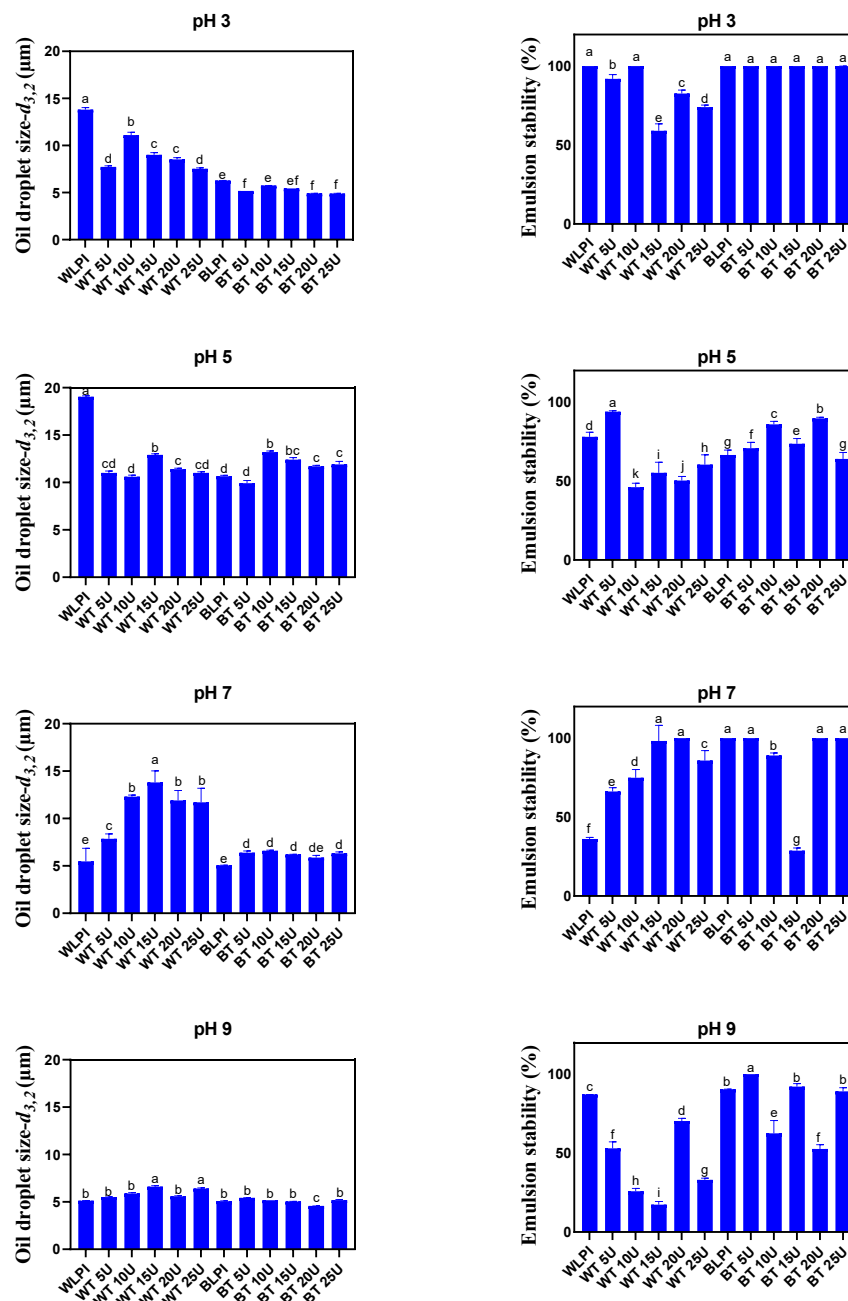


Figure 7.4 Effect of pH and enzyme concentration on emulsification capacity (A) and emulsion stability (B) of transglutaminase polymerized lupin protein isolates. Values with different superscripts within the same pH are significantly different ($p < 0.05$) as determined by one-way ANOVA and Duncan's multiple range test. WLPI-white lupin protein isolate; WT5U-white transglutaminase 5 units; WT10U-white transglutaminase 10 units; WT15U-white transglutaminase 15 units; WT20U-white transglutaminase 20 units; WT25U-white transglutaminase 25 units; BLPI-blue lupin protein isolate; BT5U-Blue transglutaminase 5 units; BT10U-Blue transglutaminase 10 units; BT15U-blue transglutaminase 15 units; BT20U-blue transglutaminase 20 units; BT25U-blue transglutaminase 25 units.

7.3.6 Emulsification capacity and emulsion stability

The oil droplet size distributions ($d_{3,2}$) and emulsion stability profiles revealed pH-dependent responses to TG treatment, with marked differences between acidic and alkaline conditions (Figure 7.4A and B). Emulsion stability is discussed on two complementary time scales. Short-term stability refers to the ability of proteins to rapidly adsorb at the oil–water interface and resist droplet growth immediately after homogenization, as reflected by changes in droplet size measured within 30 min. Longer-term physical stability, in contrast, is associated with the resistance of emulsions to droplet aggregation and fusion during storage and is evaluated using flocculation and coalescence indices after 24 h. At pH 3, progressive TG treatment resulted in a substantial reduction in droplet size, decreasing from approximately 14 μm for WLPI to 7–8 μm at 20–25 U/g. This increase in emulsification capacity under acidic conditions appears counterintuitive, considering the extensive cross-linking observed in SDS-PAGE analysis; however, it may be attributed to the formation of flexible polymeric structures with improved interfacial adsorption efficiency. Enzymatic polymerization can expose buried hydrophobic domains and increase molecular flexibility, facilitating more rapid migration and rearrangement of protein molecules at the oil–water interface (Glusac et al., 2020). However, while faster adsorption kinetics may contribute to the improved emulsification, this was not directly measured in the current study; the enhanced performance at pH 3 can be primarily attributed to interfacial film strengthening and steric stabilization provided by the cross-linked polymers. The emulsification capacity at pH 3 likely reflects the unique interfacial behavior of TG-modified proteins under acidic conditions. Cross-linked proteins may exhibit increased surface activity due to exposure of hydrophobic regions during enzymatic modification, as suggested by the structural reorganization observed in FTIR analysis. Additionally, the maintained solubility at pH 3 despite extensive cross-linking ensures sufficient protein availability for interface coverage, while the

larger molecular weight provides more effective steric stabilization once adsorbed (Damodaran & Agyare, 2013). The corresponding emulsion stability data, especially for the treated blue lupin protein isolates (100%), indicate the formation of robust protein interfacial films. Similar results at acidic pH were also obtained from other studies for soybean (Kang et al., 2024) and casein (Nogueira et al., 2019) stabilized emulsions. Although many food emulsions are formulated within pH 4–6, acidic conditions around pH 3 are highly relevant for acidified beverages, fruit-based drinks, salad dressings, and fermented products. In addition, pH 3 represents a critical stress condition near the lower limit of protein functionality, where electrostatic stabilization is challenged. The strong emulsifying performance observed at this pH demonstrates that transglutaminase modification can effectively extend the functional pH range of lupin proteins into acidic food systems. Additionally, at pH 5, near the isoelectric point, TG treatment exhibited divergent effects between the two protein types. For WLPI, enzyme modification improved emulsifying performance, reducing droplet sizes from approximately 18 μm (native protein-stabilized emulsion) to 9-12 μm across all enzyme concentrations, representing a 35-50% improvement. In contrast, BLPI showed reduced performance following TG treatment, with droplet sizes increasing from the native protein's smaller droplet size (10 μm) to larger sizes at all enzyme concentrations tested (10-14 μm). This species-specific divergence at pH 5 directly correlates with their different solubility results (Table 7.1), with BLPI showing complete insolubilization at higher enzyme concentrations, while WLPI maintained partial solubility. The limited effectiveness of TG-modified proteins at pH 5, particularly for BLPI, is constrained by intrinsic protein solubility rather than by insufficient enzymatic cross-linking. SDS-PAGE analysis (Figure 7.2) confirmed that TG catalyzed cross-linking during the modification step; however, when these cross-linked proteins are dispersed at pH 5, the high-molecular-weight aggregates

exhibit poor solubility near the isoelectric point, leading to precipitation and reduced protein availability at the oil-water interface. The minimal electrostatic repulsion at the isoelectric point (Figure 7.1) eliminates charge-based stabilization, requiring greater reliance on steric effects and protein availability at interfaces. For BLPI, the severe aggregation and precipitation at pH 5 limit protein availability for interface coverage, whereas the polymerized WLPI retained partial solubility, enabling continued, albeit reduced, emulsification capacity (Jiang et al., 2019). At pH 7, both native proteins demonstrated good emulsifying capability with small droplet sizes ($\sim 5 \mu\text{m}$), but TG treatment induced contrasting effects. WLPI exhibited an unexpected deterioration, with droplet sizes increasing from approximately $5 \mu\text{m}$ (native) to $8\text{-}12 \mu\text{m}$ across all enzyme concentrations, resulting in a 60-140% increase in droplet diameter. Despite this increase in droplet size, emulsion stability remained high, suggesting that while initial droplet formation was impaired, the emulsified oil droplets were stable after 30 min. BLPI demonstrated better tolerance to enzymatic modification, with only a slight increase from $\sim 5 \mu\text{m}$ (native) to about $6 \mu\text{m}$ at all enzyme concentrations. The deterioration in WLPI's droplet formation ability at pH 7, despite moderate solubility, suggests that cross-linking may reduce the rate of protein adsorption or interfacial rearrangement necessary for efficient droplet breakup during homogenization (Nivala et al., 2021). At pH 9, TG treatment had minimal impact on emulsifying performance, with all samples (both native and modified) producing uniform, small droplets in the range of $4\text{-}6 \mu\text{m}$. This consistency across all conditions, regardless of protein type or enzyme concentration, indicates that the strong negative charge at pH 9 (-20 to -30 mV , Figure 1) may dominate the emulsification mechanism. The high electrostatic repulsion enables efficient droplet breakup during homogenization and prevents coalescence. This effectively masks any potential negative effects of cross-linking on interfacial properties. The high solubility at pH 9 (Table 7.1) also ensures

sufficient protein availability for rapid interface coverage. The results suggest that alkaline conditions represent the most optimal operating range for TG-modified lupin proteins, as the dominant electrostatic effects override any structural modifications that might otherwise compromise emulsifying performance (San et al., 2025). These findings suggest that TG modification can significantly enhance the emulsifying properties of lupin protein, but success requires careful control of both enzyme concentration and system pH to balance the benefits of cross-linking against solubility limitations.

7.3.7 Microstructure of emulsions

The CLSM images provide direct visual evidence of emulsion microstructure changes induced by TG treatment across the pH spectrum, with distinct protein-oil interfacial arrangements visible through dual fluorescence labeling (Figure 7.5). At pH 3, both native proteins (WLPI and BLPI) exhibited heterogeneous droplet size distributions, characterized by numerous large droplets (yellow/orange fluorescence indicating oil). In contrast, WT5U and BT5U showed markedly improved homogeneity, with predominantly smaller, uniformly distributed droplets. This visual confirmation corroborates the quantitative reduction in droplet size observed at pH 3 (Figure 7.4A). The result demonstrates that TG modification enhances droplet breakup during homogenization and prevents coalescence through improved interfacial stabilization (Kamiya et al., 2009). The green fluorescence surrounding oil droplets, representing FITC-labeled proteins, appeared more intense and continuous in enzyme-treated samples, suggesting formation of thicker, more cohesive protein films at the oil-water interface following cross-linking (Kamiya et al., 2009). At pH 5, the result revealed differences between protein types that align with their emulsifying behaviors. WLPI samples maintained relatively dispersed droplet structures even after enzyme treatment (WT5U), with visible protein coverage (green) around oil droplets, consistent

with the improved droplet sizes measured quantitatively. In contrast, BLPI and BT5U exhibited extensive droplet aggregation and coalescence, appearing as large irregular oil domains with incomplete protein coverage, visually confirming the poor emulsifying performance at the isoelectric point (Tang, 2017). The reduced green fluorescence intensity in BT5U samples suggests limited protein availability at interfaces due to the extensive precipitation observed in solubility studies (Table 7.1), leaving oil droplets inadequately protected against coalescence (Tang, 2017). The pH 7 images revealed microstructural features that further explain the quantitative results. Despite WLPI showing increased droplet sizes with treatment (Figure 7.4A), the WT5U sample displayed extensive protein coverage (bright green fluorescence) throughout the continuous phase and at droplet interfaces. This suggests that while cross-linking may impair the initial emulsification process (resulting in larger droplets), it creates protein networks that could provide long-term stability through formation of a gel-like matrix between droplet (Kamiya et al., 2009). The BLPI samples maintained more discrete droplet structures at pH 7, with clear protein layers surrounding individual oil droplets in both native and treated samples, supporting the minimal size changes observed in Figure 7.4A. At pH 9, all samples exhibited uniform microstructures characterized by small, well-separated droplets with distinct protein interfacial layers, regardless of TG treatments. The bright, continuous green fluorescence throughout the aqueous phase indicates high protein solubility, ensuring abundant material for interface coverage even in heavily cross-linked samples. The CLSM analysis reveals that TG modification not only affects droplet size but also alters the protein organization within emulsions. At pH values with sufficient charge (pH 3 and 9), enzyme treatment enhances interfacial film thickness without compromising droplet dispersion. However, near the isoelectric point (pH 5), species-specific aggregation behaviors

become visually apparent, with BLPI showing coalescence while WLPI maintains slight functionality.

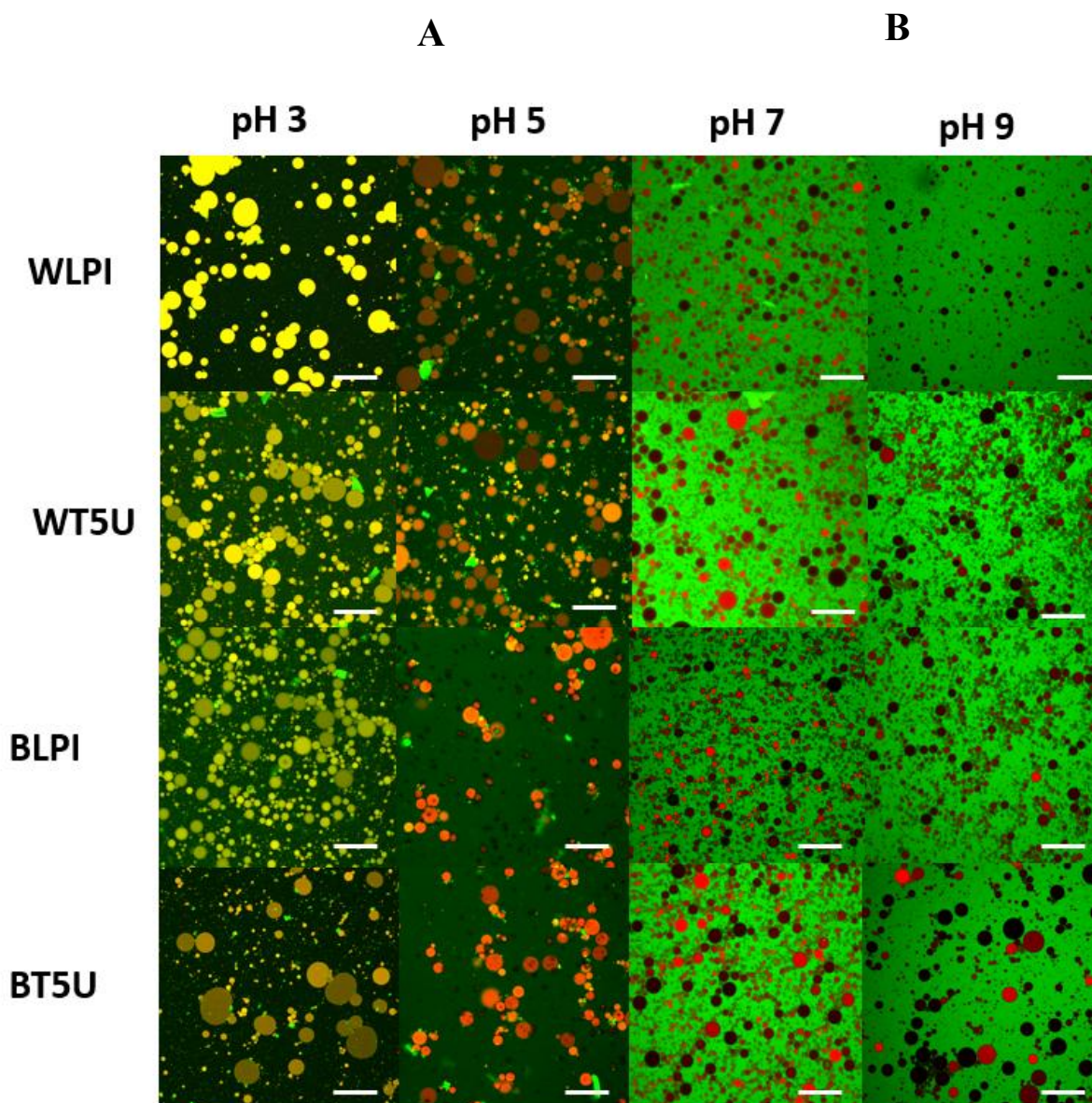


Figure 7.5 Confocal laser scanning microscopy of lupin protein isolates and transglutaminase polymerized lupin protein isolates. The scale bars within the figures represent 40 μm in length. WLPI-white lupin protein isolate; WT5U-white transglutaminase 5 units; BLPI-blue lupin protein isolate; BT5U-blue transglutaminase 5 units.

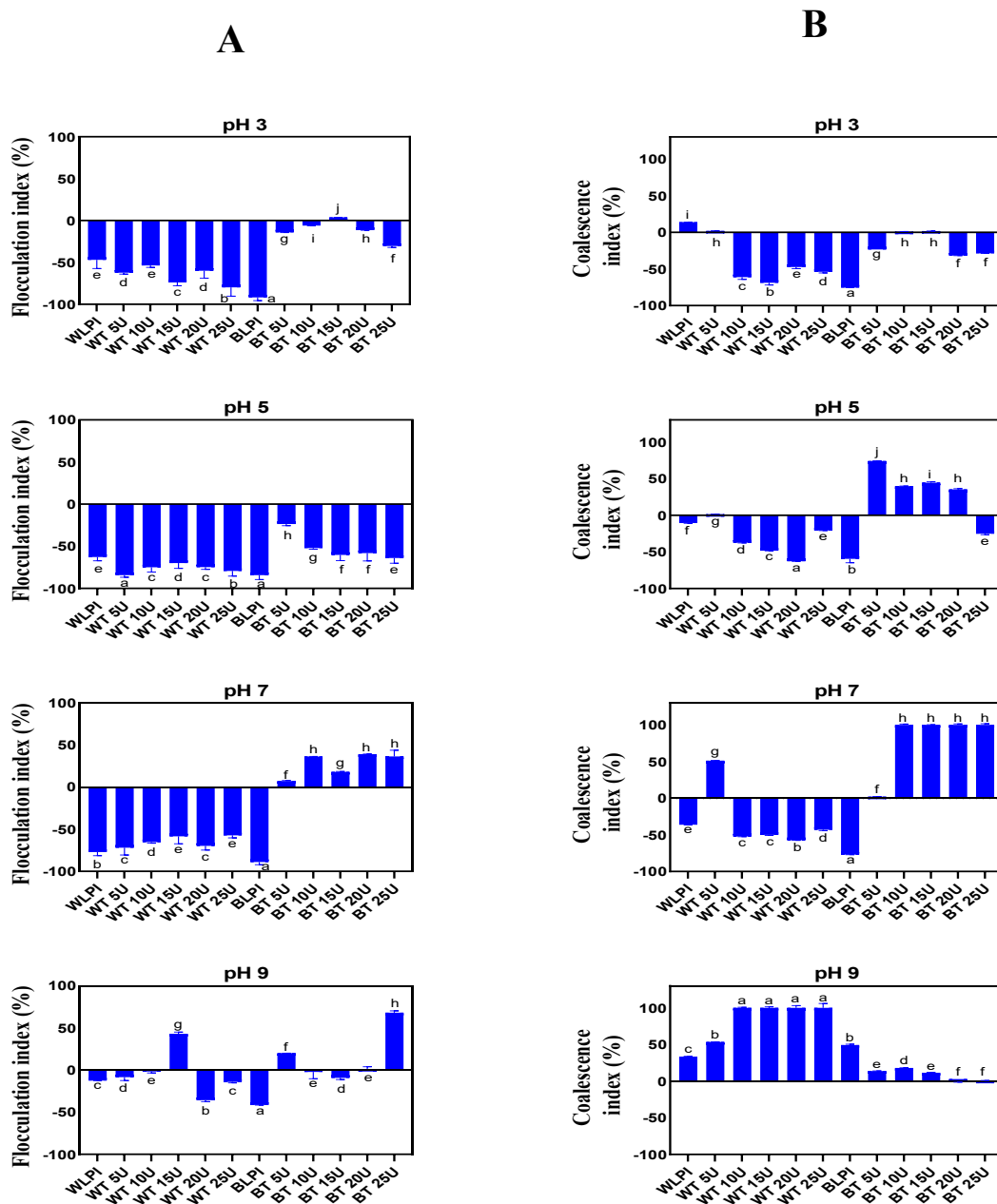


Figure 7.6 Effect of pH and enzyme concentration on A: flocculation index and B: coalescence index of transglutaminase polymerized lupin protein isolates. Values with different superscripts within the same pH are significantly different ($p < 0.05$) as determined by one-way ANOVA and Duncan's multiple range test. WLPI-white lupin protein isolate; WT5U-white transglutaminase 5 units; WT10U-white transglutaminase 10 units; WT15U-white transglutaminase 15 units; WT20U-white transglutaminase 20 units; WT25U-white transglutaminase 25 units; BLPI-blue lupin protein isolate; BT5U-Blue transglutaminase 5 units; BT10U-Blue transglutaminase 10 units; BT15U-blue transglutaminase 15 units; BT20U-blue transglutaminase 20 units; BT25U-blue transglutaminase 25 units.

7.3.8 Flocculation and coalescence indices

Flocculation represents the reversible aggregation of droplets into clusters, whereas coalescence involves the irreversible fusion of droplets and subsequent phase separation. Both processes are strongly pH-dependent, with coalescence stability depending on the strength and integrity of the interfacial protein film. The flocculation (FI) and coalescence indices (CI) revealed pH-dependent emulsion destabilization mechanisms that directly correlated with surface charge and enzymatic cross-linking effects (Figure 7.6). At pH 3, native proteins (WLPI and BLPI) displayed predominantly negative FI values, indicating limited droplet aggregation due to strong electrostatic repulsion from positively charged protein surfaces. However, TG-treated samples exhibited markedly increased FI values, suggesting that enzymatic cross-linking promoted bridging flocculation between droplets. This phenomenon mirrors observations in whey protein systems, where TG treatment enhanced droplet interactions through formation of protein networks that physically connected adjacent droplets, creating a gel-like structure that stabilized against creaming despite apparent flocculation (Palazolo et al., 2005). Importantly, CI values remained low at pH 3 for all samples, confirming that while droplets may cluster through flocculation, the robust cross-linked interfacial films effectively prevented irreversible coalescence, a critical parameter for emulsion stability. At pH 5, both protein types exhibited elevated FI and CI values, with BLPI showing instability. Near the isoelectric point, the near-zero net surface charge eliminated electrostatic repulsion, enabling rapid droplet aggregation followed by coalescence. The simultaneous increase in both FI and CI at pH 5 indicates a two-stage destabilization process: initial flocculation driven by the abolition of electrostatic repulsion, followed by coalescence as attractive van der Waals forces dominate over weakened steric barriers (Tran & Rousseau, 2013). Research on pea protein emulsions demonstrated similar pH-dependent behavior, with maximum instability occurring near the pI where protein aggregation compromised interfacial protection

(Opazo-Navarette et al., 2022). The pH 7 results revealed species-specific differences. BLPI maintained relatively low FI and CI values, suggesting stable dispersions consistent with adequate electrostatic repulsion at neutral pH. However, WT5U showed elevated FI with low CI, indicating controlled flocculation without coalescence, potentially representing formation of stable flocculated networks. Studies on pork myofibrillar protein have demonstrated that TG treatment at neutral pH improves emulsion stability by creating cross-linked protein networks that enhance creaming stability, despite visible flocculation. This supports the current observation that flocculation does not necessarily indicate instability when interfacial films remain intact (Chukwuejim & Aluko, 2024). The increased CI in some samples at pH 7 may reflect compromised interfacial integrity where cross-linking impaired protein flexibility needed for effective interfacial stabilization. At pH 9, FI values fluctuated between positive and negative values across all samples, while CI values exhibited substantial variation. The high negative surface charge (-20 to -30 mV) provided strong electrostatic stabilization, preventing coalescence even when moderate flocculation occurred. Research on faba bean TG-modified proteins has demonstrated that pH 6.5 and above, there is enhanced cross-linking efficiency and emulsion stability, consistent with current observation that enzymatic treatment at pH 9 maintains low coalescence despite flocculation (Nivala et al., 2021). These findings demonstrate that emulsion stability depends on the balance between flocculation tendency and coalescence resistance, with pH modulating both electrostatic and steric stabilization mechanisms. TG cross-linking enhanced interfacial film strength, reducing coalescence particularly at pH values where electrostatic repulsion was moderate; however, its effectiveness was limited near the isoelectric point, where fundamental charge-based stability was absent.

7.4 CONCLUSIONS

This work established that TG modification represents a viable enzymatic approach for enhancing the emulsifying properties of lupin protein isolates, though effectiveness depends critically on species selection, enzyme concentration, and pH conditions. The formation of covalent ϵ -(γ -glutamyl)lysine cross-links successfully generated high-molecular-weight polymers with altered secondary structures, transitioning from predominantly β -sheet conformations toward increased α -helical content at moderate enzyme concentrations. However, extensive cross-linking at higher enzyme levels (20-25 U/g) produced aggregated β -sheet structures that compromised solubility and functionality, particularly near the isoelectric point. The superior performance of TG-modified proteins at pH 3, characterized by substantial reductions in droplet size and enhanced stability, suggests their particular suitability for acidic emulsified food systems such as salad dressings, acidified beverages, and fermented dairy alternatives. At pH 9, the dominance of electrostatic stabilization mechanisms rendered enzymatic modifications largely redundant, although protein solubility and availability remained excellent. The microstructural analysis revealed that TG treatment alters protein organization within emulsions, increasing interfacial film thickness and forming continuous-phase networks that provide complementary stabilization mechanisms. The decoupling of flocculation from coalescence at certain pH values demonstrates that controlled droplet aggregation through protein network formation does not necessarily compromise emulsion stability when interfacial films remain intact. However, it should be noted that TG-modified lupin proteins exhibit significant limitations near the isoelectric region (pH 4-6), where reduced solubility and precipitation of cross-linked aggregates compromise their emulsifying functionality; therefore, applications in this pH range are not recommended. These findings provide a rational framework for optimizing TG treatment conditions, suggesting that moderate enzyme concentrations (10-15 U/g) applied to white lupin proteins represent the most promising approach

for developing enhanced plant-based emulsifiers with high performance at acidic or alkaline pH. Future research should explore the long-term storage stability, thermal processing resistance, and sensory attributes of food products formulated with these modified proteins to facilitate their commercial implementation in sustainable, plant-based food systems.

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ETHICAL STATEMENT

Studies in humans and animals. The authors declare that the research presented does not involve any animal or human study.

AUTHORSHIP

R.E. Aluko Conceived, designed, supervised, reviewed, and edited the manuscript's final draft. S. Chukwuejim carried out the research, analyzed the data, and wrote the first draft of the manuscript.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The author is an Editorial Board Member/Editor-in-Chief/Associate Editor/Guest Editor for Journal of Functional Foods, Food Chemistry, and Food Chemistry X and was not involved in the editorial review or the decision to publish this article.

DATA AVAILABILITY

The data from the current study are available from the corresponding author and first author of the article on reasonable request.

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TRANSITION STATEMENT

Chapters 3 through 7 collectively addressed the first research stream of this thesis: understanding and improving the techno-functional properties of lupin proteins. Chapter 7 (Manuscript 6) concluded this stream by demonstrating that transglutaminase-catalyzed cross-linking is a viable enzymatic strategy for enhancing the emulsifying performance of both white and blue lupin protein isolates. Moderate enzyme concentrations (10–15 U/g) applied to white lupin protein isolate yielded a favorable balance between polymerization and retained solubility, producing smaller oil droplets, thicker interfacial films, and continuous-phase protein networks that conferred stabilization in both the interfacial and bulk phases. These findings, combined with the native characterization data from earlier chapters, provide a robust framework for selecting and modifying lupin protein ingredients for targeted food applications. The thesis now transitions to its second research stream: the discovery of bioactive peptides from lupin proteins. Whereas Chapters 3–7 focused on the intact protein and its enzymatically cross-linked derivatives, Chapter 8 (Manuscript 7) employs a complementary enzymatic approach and controlled proteolytic hydrolysis to break lupin proteins into smaller peptide fragments and screen them for a specific biological activity: inhibition of arginase. Arginase dysregulation is implicated in cardiovascular diseases, erectile dysfunction, cancer, and inflammatory conditions, yet food-derived arginase inhibitors remain virtually unexplored. Chapter 8 systematically evaluated hydrolysates produced from white and blue lupin protein isolates using multiple proteases, identified the most potent arginase-inhibitory fractions by reverse-phase HPLC, determined peptide sequences by tandem mass spectrometry, and characterized the inhibitory kinetics and molecular binding interactions of the most active peptides through kinetic studies, molecular docking, and molecular dynamics simulations.

CHAPTER EIGHT

MANUSCRIPT SEVEN

KINETICS AND MOLECULAR DOCKING STUDIES OF THE INHIBITION OF ARGINASE BY LUPIN SEED PROTEIN-DERIVED PEPTIDES

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ABSTRACT

Arginase dysregulation is implicated in various pathological conditions, including cardiovascular diseases, erectile dysfunction, and cancer, making it an attractive therapeutic target. In this study, enzymatic hydrolysates of white lupin (*Lupinus albus*) and blue lupin (*Lupinus angustifolius*) protein isolates were evaluated for arginase-inhibitory activity. Among the hydrolysates tested, white lupin flavourzyme hydrolysate exhibited the highest inhibitory potency (~35% at 800 µg/mL), followed by trypsin and pepsin hydrolysates, while alcalase hydrolysates showed no activity. Reverse-phase HPLC fractionation followed by tandem mass spectrometry identified ten peptide sequences from the most active fractions. Two structurally distinct peptides, the short hydrophobic hexapeptide DMGAVA and the long polycationic 16-mer VVALKKVGVLKKPGPT, demonstrated the highest arginase inhibition and were selected for mechanistic studies. Lineweaver-Burk analysis revealed mixed-type inhibition kinetics with distinct profiles: DMGAVA substantially affected both K_m and V_{max} ($K_i \sim 266 \mu M$), whereas VVALKKVGVLKKPGPT showed predominantly competitive behavior with minimal change in V_{max} ($K_i \sim 589 \mu M$).

KEYWORDS: Lupin, peptides, arginase, molecular docking, molecular dynamics, enzyme inhibition

8.1 INTRODUCTION

Arginases are manganese-containing metalloenzymes that catalyze the hydrolysis of L-arginine to produce urea and L-ornithine (Wu & Morris, 1998). These enzymes play critical roles in several physiological processes, including elimination of ammonia from the body through the urea cycle, production of polyamines and proline that are essential for cell proliferation and collagen synthesis, and regulation of nitric oxide (NO) bioavailability by competing with nitric oxide synthase (NOS) for their shared substrate, L-arginine (Morris, 2009). Two isoforms of arginase exist in mammals: arginase I, a cytosolic enzyme predominantly expressed in the liver, and arginase II, a mitochondrial enzyme primarily found in the kidney but also present in various other tissues (Caldwell et al., 2018). Both isoforms are implicated in the pathophysiology of numerous diseases, including cardiovascular disorders such as hypertension and atherosclerosis, erectile dysfunction, pulmonary diseases, and cancer (Bivalacqua et al., 2001; Canè et al., 2025; Xu et al., 2004).

The therapeutic potential of arginase inhibition has garnered significant attention due to the enzyme's involvement in various pathological conditions. In cardiovascular diseases, elevated arginase activity reduces NO bioavailability, leading to endothelial dysfunction, vasoconstriction, and hypertension (Caldwell et al., 2018). Similarly, in erectile dysfunction, increased arginase expression in the corpus cavernosum diminishes NO-mediated smooth muscle relaxation, impairing penile erection (Abdel Wahed et al., 2018). In cancer, arginase overexpression in tumor-associated myeloid cells depletes L-arginine from the tumor microenvironment, suppressing T-cell proliferation and antitumor immune responses (Niu et al., 2022). Consequently, arginase inhibitors have emerged as promising therapeutic agents for managing these conditions. Several synthetic arginase inhibitors have been developed over the past decades, including N ω -hydroxy-L-arginine (nor-NOHA), 2(S)-amino-6-boronoheptanoic acid (ABH), S-(2-boronoethyl)-L-

cysteine (BEC), and INCB001158 (CB-1158) (Grobben et al., 2020; Pudlo et al., 2017). These compounds primarily target the conserved active site of arginases and compete with L-arginine for binding. However, despite their potent *in vitro* inhibitory activity, these synthetic inhibitors face significant limitations that have hindered their clinical translation. Key challenges include poor pharmacokinetic profiles, characterized by low oral bioavailability, rapid metabolic degradation, and unfavorable absorption, distribution, metabolism, and excretion (ADME) properties (Pudlo et al., 2017). Additionally, the highly conserved active sites of arginase I and II pose challenges for developing isoform-selective inhibitors, which may be necessary to target specific disease pathologies while minimizing off-target effects (Gao et al., 2021). CB-1158 (INCB001158) and OATD-02, only entered Phase I/II clinical trials for cancer immunotherapy in late 2019 and 2022, respectively, underscoring the slow progress in this field (Grzybowski et al., 2022; Naing et al., 2024; Papadopoulos et al., 2017). These limitations have prompted the search for alternative arginase inhibitors from natural sources, particularly food-derived bioactive peptides.

Food-derived bioactive peptides have attracted considerable interest as potential enzyme inhibitors due to their generally recognized safety, structural diversity, and potential for multifunctional bioactivities (Udenigwe & Aluko, 2012). These peptides are typically released from parent proteins through enzymatic hydrolysis, gastrointestinal digestion, or microbial fermentation, and have demonstrated inhibitory activities against various enzymes of physiological relevance, including angiotensin-converting enzyme (ACE), dipeptidyl peptidase-IV (DPP-IV), and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) (Cruz-Chamorro, Santos-Sánchez, Álvarez-López, et al., 2023; Zhao et al., 2020). Notably, a recent study demonstrated that a synthetic peptide (REGNH) designed based on the C-terminal S-shaped motif of human arginase I exhibited non-competitive inhibition of both arginase I and II, with IC₅₀ values

of 2.4 ± 0.3 mM and 1.8 ± 0.1 mmol, respectively (Gao et al., 2021). This finding suggests that peptide-based compounds can modulate arginase activity through mechanisms distinct from active-site competition, thereby providing a promising avenue for developing novel arginase inhibitors.

Lupin seeds (*Lupinus* spp.) are a valuable source of high-quality plant proteins that can be hydrolyzed to generate bioactive peptides. The two most commercially cultivated species, white lupin (*Lupinus albus*) and narrow-leaved lupin (*Lupinus angustifolius*), contain approximately 33–47% protein on a dry weight basis, predominantly comprising the storage proteins conglutin α , β , γ , and δ (Chukwuejim et al., 2023). Enzymatic hydrolysis of lupin protein isolates has been shown to release peptides with diverse biological activities, including antioxidant, immunomodulatory, hypotensive, hypoglycemic, and hypolipidemic effects (Cruz-Chamorro, Santos-Sánchez, Álvarez-López, et al., 2023). Particularly relevant to enzyme inhibition, lupin protein hydrolysates have demonstrated significant inhibitory activities against ACE, DPP-IV, and HMG-CoA reductase both *in vitro* and *in vivo* (Cruz-Chamorro, Santos-Sánchez, Bollati, et al., 2023; Li et al., 2025; Santos-Sánchez et al., 2022). Despite the extensive research on the enzyme-inhibitory properties of lupin-derived peptides against targets such as ACE, DPP-IV, and HMG-CoA reductase, there remains a notable paucity of information regarding peptides with arginase-inhibitory properties from lupin or other food sources. Given the therapeutic relevance of arginase inhibition in cardiovascular diseases, erectile dysfunction, and cancer, and the limitations of existing synthetic inhibitors, exploring lupin seeds as a source of arginase-inhibitory peptides represents a promising, largely unexplored avenue of research. The high protein content and established bioactivity of lupin-derived peptides provide a strong rationale for investigating their potential to modulate arginase activity. In the current work, protein hydrolysates from *Lupinus*

albus and *Lupinus angustifolius* were fractionated by reverse-phase HPLC, followed by tandem mass spectrometry to identify peptide sequences in the most potent arginase-inhibitory fractions. These peptides were synthesized and studied for the kinetics of arginase inhibition, and molecular interactions involved in maintaining the enzyme–peptide complexes through molecular docking and molecular dynamics simulations

8.2 MATERIALS AND METHODS

8.2.1 Materials

The Lupin Platform Inc. (Calgary, AB, Canada) donated blue (*Lupinus angustifolius* ‘Boregine’) and white (*Lupinus albus* ‘Dieta’) lupin seeds. Chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4), pancreatin (EC Number 232-468-9), pepsin (EC 3.4.23.1), alcalase (EC 3.4.21.62), and L-arginase from bovine liver (EC 3.5.3.1) were sourced from Millipore Sigma (St. Louis, MO, USA). Methanol (HPLC grade), trifluoroacetic acid, L-arginine, glycine, and 0.45- and 0.2- μ m syringe filters were sourced from Fisher Scientific Company (Oakville, ON, Canada). Protease from *Aspergillus oryzae* (flavourzyme EC 3.4.11.1) was sourced from TCI AMERICA (Harborside, Portland, USA). Peptides identified from mass spectrometry were synthesized *de novo* at GenScript Inc. (Piscataway, NJ, USA) with purities exceeding 95%. All other reagents and chemicals utilized in this study were of high-purity analytical grade. The reagents and chemicals were prepared using double-distilled water (DDW).

8.2.2 Preparation of lupin protein hydrolysates

Blue lupin protein isolate (BLPI) and white lupin protein isolate (WLPI) were prepared following a previously described procedure (Chukwuejim & Aluko, 2025). For enzymatic hydrolysis, lupin protein isolates were suspended in distilled water at 5% (w/v) and adjusted to the optimal pH and temperature for each proteolytic enzyme. Hydrolysis was initiated by adding the respective

enzyme once optimal conditions were reached: alcalase (50°C, pH 8.0), pepsin (37°C, pH 2.0), trypsin (37°C, pH 8.0), chymotrypsin (37°C, pH 8.0), and flavourzyme (50°C, pH 8.0). For the sequential pepsin-pancreatin digestion, the protein isolate was first dispersed in 5% distilled water and incubated with pepsin at pH 2.0 for 2 h. Subsequently, the mixture was adjusted to pH 7.5 using 2 M NaOH, and pancreatin added for an additional 2 h incubation. Aliquots (2 mL) were withdrawn from the reaction mixture at 30-min intervals to monitor the degree of hydrolysis (DH). After 4 h, the reaction was terminated by adjusting the mixture to pH 4.5 with 2 M HCl, followed by heat inactivation in a boiling water bath (100°C) for 15 min to ensure complete enzyme denaturation. The digests were subsequently cooled to 20°C using an ice bath. The cooled samples were centrifuged at $10,000 \times g$ for 30 min, and the resulting supernatants collected, lyophilized, and stored at -20°C as lupin protein hydrolysates (LPH). The degree of hydrolysis was quantified using the o-phthaldialdehyde (OPA) assay, as described by Nielsen et al. (2001).

8.2.3 LPH fractionation by reversed-phase high-pressure chromatography (RP-HPLC)

LPH was fractionated using RP-HPLC on a preparative C12 column (250 mm $\times$ 21 mm, 10 μ m particle size; Phenomenex Inc., Torrance, CA, USA) connected to a Shimadzu HPLC system (Shimadzu Scientific Instruments Inc., MD, USA) equipped with a photodiode array UV-VIS detector and an autosampler. The separation protocol was adapted from Zhang et al. (2018) with minor modifications. Lyophilized LPH was reconstituted in double-distilled water containing 0.1% trifluoroacetic acid (TFA; mobile phase A) to achieve a final concentration of 100 mg/mL. The sample solution was sequentially filtered through 0.45 μ m and 0.2 μ m membrane filters prior to injection. A 2 mL aliquot of the filtered sample was loaded onto the C12 column, and peptides were eluted at a flow rate of 5 mL/min using a linear gradient of 0–100% mobile phase B (methanol containing 0.1% TFA) over 60 min. Peptide elution was monitored at 214 nm, and eluted peptides

collected automatically and pooled into seven fractions designated F1, F2, F3, F4, F5, F6, and F7. The organic solvent was removed from each pooled fraction using a vacuum rotary evaporator maintained at 40°C, and the resulting aqueous phase was lyophilized. All fractions were subsequently evaluated for arginase I inhibitory activity. The fraction exhibiting the highest inhibitory activity (F1) was subjected to a second RP-HPLC separation. For this purification step, 100 mg/mL of F1 was loaded onto the same C12 preparative column using identical mobile phases, flow rate, and detection wavelength as the initial separation. Peptides were eluted using a narrower linear gradient of 16–25% mobile phase B over 20 min. Seven sub-fractions (F1-1, F1-2, F1-3, F1-4, F1-5, F1-6, and F1-7) were collected using an automated fraction collector. The organic solvent was evaporated from each sub-fraction using vacuum rotary evaporator maintained at 40°C, and the aqueous residues were lyophilized.

8.2.4 Mass spectrometry and peptide sequence identification

The HPLC fractions demonstrating the highest inhibitory activity against arginase I following the second round of chromatographic separation (F1-1, F1-5, F1-6, and F1-7) were subjected to tandem mass spectrometry analysis for peptide identification. Samples were prepared at a concentration of 10 ng/μL in 0.1% aqueous formic acid and introduced into an AB Sciex QTRAP 6500 mass spectrometer (AB Sciex Ltd., Foster City, CA, USA) equipped with an electrospray ionization (ESI) source. The instrument was operated under the following conditions: ion spray voltage of 5.5 kV, source temperature of 200°C, curtain gas pressure of 30 psi, ion-source gas 1 pressure of 50 psi, ion-source gas 2 pressure of 60 psi, and collision gas set to high. Analysis was performed in positive ion mode at a flow rate of 30 μL/min over a 10-min acquisition period, scanning a mass-to-charge (m/z) range of 70–1000. The resulting MS/MS spectra were processed using PEAKS 7.0 Studio software (Bioinformatics Solutions, Waterloo, ON, Canada) to elucidate

peptide sequences. Peptides identified through this analysis were subsequently custom synthesized by Genscript Inc. (Piscataway, NJ, USA) with >95% purity.

8.2.5 Arginase I inhibition and kinetic analysis

Arginase I inhibition was evaluated by quantifying urea production using previously established colorimetric methods (Gao et al., 2021), with minor modifications. Assays were conducted in 96-well microplates using assay buffer comprising 100 mM sodium phosphate (pH 7.4) and 130 mM NaCl. Each reaction had a final volume of 250 μ L with Arginase I (50 μ L) at assay concentration of 7 units/mL. Negative control wells contained no enzyme. Peptide inhibitors (50 μ L) were added at concentrations ranging from 10 μ g/mL to 100 μ g/mL. Samples were pre-incubated for 30 min at 37°C with shaking at 100 rpm. Enzymatic reactions were initiated by adding the substrate solution (125 μ L), consisting of assay buffer supplemented with 100 mM L-arginine, 500 mM glycine, and 1.25 mM MnCl₂. The enzymatic reactions proceeded at 37°C with continuous shaking and were terminated after 1 h by adding a stop mix solution. The stop mix was freshly prepared by combining two volumes of solution A (antipyrine/H₂SO₄ reagent: 5 g/L antipyrine [1,5-dimethyl-2-phenyl-3-pyrazolone] dissolved in 50% [v/v] sulfuric acid) with one volume of solution B (oxime reagent: 0.8 g 2,3-butanedione monoxime dissolved in 100 mL of 5% [v/v] acetic acid). Following quenching, the reaction mixtures were incubated in the dark at room temperature for at least 18 h to allow color development, then heated at 45°C in a water bath for 4 h. The resulting brown coloration, corresponding to urea production, was quantified by measuring optical density at 466 nm using a Synergy H1 Hybrid microplate reader (BioTek Instruments, Winooski, VT, USA). Kinetic measurements were further performed at a series of L-arginine concentrations (12.5–400 mM). The initial velocities were modeled using the Michaelis–Menten

equation, and Lineweaver–Burk plots were generated with GraphPad Prism 10.0 (GraphPad Software, Boston, MA, USA) to estimate apparent K_m , V_{max} , and inhibition constant (K_i).

8.2.6 Molecular docking of the synthesized peptides

The crystal structure of human arginase I (PDB: 2AEB) was obtained through the UniProt database (www.uniprot.org) and cleaned using PyMOL v2.0.7. The docking was performed as previously described by Fatoki et al. (2023). Briefly, the two cleaned arginase structures in .pdb format were docked with each of the peptide sequences (DMGAVA and VVALKKVGVLKKPGPT) in FASTA format, respectively, using the HPEPDOCK 2.0 webserver (Zhou et al., 2018), available at (<http://huanglab.phys.hust.edu.cn/hpepdock/>). The top-ranked model was used to build each peptide-protein complex using PyMOL v2.0.7, and the docking binding affinity and dissociation constant (K_d) were analyzed using the PRODIGY webserver (Xue et al., 2016), available at (<https://rascar.science.uu.nl/prodigy/>). The 3D structure of the complex was visualized using PyMOL, and the 2D structure was visualized using the PLIP webserver (Schake et al., 2025) and LigPlot⁺ version 2.3.1 (Laskowski & Swindells, 2011)

8.2.7 Molecular dynamics (MD) of the synthesized peptides

MD simulations were employed to study the conformational stability of the peptide-protein complexes formed by the DMGAVA and VVALKKVGVLKKPGPT peptides, respectively, bound to human arginase I (PDB: 2AEB). Initial preparation of each peptide-protein complex was done using CHARMM-GUI webserver available at (<https://www.charmm-gui.org/>) (Jo et al., 2008; J. Lee et al., 2016). The simulation box size was extended enough to cover each atom of the protein, and the box was greater than 1.0 nm. The TIP3P water model was used to solvate the peptide-protein complex, and the solution was neutralized with 0.15 M Na^+ and Cl^- ions. Energy optimization was performed using a maximum force of $1000 \text{ kJ}\cdot\text{mol}^{-1}\cdot\text{nm}^{-1}$ via the steepest-descent method. To

stabilize the system temperature and pressure, equilibration was performed in the NVT (constant number of particles, volume, and temperature) ensemble at 300 K for 125 ps, and in the NPT (constant number of particles, pressure, and temperature) ensemble at 1 bar and 125 ps. Solvated peptide-protein complexes were run for 100 ns in production MD. GROMACS version 2024.4 (Abraham et al., 2015) was used to perform energy minimization, NVT and NPT equilibration, and the final production steps of MD simulations using the CHARMM36-JUL2022 force field (Best et al., 2012). Temperature and pressure were controlled using V-rescale and Parrinello-Rahman methods, respectively. After the MD simulation, trajectories were analyzed, and binding free energies were calculated using the Molecular Mechanics-Generalized Born Surface Area (MMGBSA) method with the gmx_MMPBSA tool for GROMACS files (Valdés-Tresanco et al., 2021)

8.2.8 Statistical analysis

All experiments were performed in triplicate, and results are reported as mean $\pm$ standard deviation. Statistical differences among treatments were evaluated using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test at a significance level of $p \leq 0.05$. Statistical analyses were conducted using IBM SPSS Statistics for Windows (Version 26.0), and graphical representations were generated using GraphPad Prism (Version 10).

8.3 RESULTS AND DISCUSSION

8.3.1 Arginase Inhibitory Activity of Lupin Protein Hydrolysates

The arginase inhibitory activities of enzymatic hydrolysates from white lupin (*Lupinus albus*) and blue lupin (*Lupinus angustifolius*) proteins are presented in Figure 8.1A. All hydrolysates exhibited dose-dependent inhibition, with activity increasing progressively at 400, 600, and 800 $\mu\text{g/mL}$. Among the hydrolysates tested, considerable variations in arginase inhibitory activity were

observed, which can be attributed to differences in the proteolytic specificity of the enzymes employed and the resultant peptide profiles. The white lupin flavourzyme hydrolysate (WFLA) exhibited the highest arginase inhibitory activity (~35% at 800 µg/mL), followed by white lupin trypsin hydrolysate (WTRP, ~32%). In contrast, alcalase and pepsin-pancreatin hydrolysates from both lupin species showed no detectable arginase inhibitory activity. These findings are consistent with previous studies demonstrating that the choice of protease significantly influences the biological activity of the resulting peptides, owing to their distinct cleavage specificities and the consequent generation of peptides with varying amino acid compositions and chain lengths (Danilova & Sharipova, 2020; Gurumalles et al., 2019). The superior performance of the flavourzyme hydrolysate is noteworthy, as flavourzyme is a fungal protease complex containing both endo- and exopeptidase activities, which generates peptides with diverse chain lengths that may enhance enzyme binding interactions (Lee et al., 2023). The strong inhibitory activity of trypsin hydrolysates suggests that peptides with C-terminal lysine or arginine residues, characteristic of tryptic cleavage, may be particularly effective arginase inhibitors. This is mechanistically plausible given that arginase naturally binds the positively charged guanidinium group of L-arginine, and peptides with basic C-terminal residues may mimic substrate interactions (Yan et al., 2025). The moderate activity of pepsin hydrolysates indicates that gastrointestinal digestion conditions can also release arginase-inhibitory peptides, which have implications for the bioavailability of these peptides following oral consumption (Ashaolu et al., 2024).

Interestingly, white lupin hydrolysates consistently exhibited higher arginase inhibitory activity than their blue lupin counterparts across all enzyme treatments. This species-dependent variation may be related to differences in the primary structure and amino acid composition of the major storage proteins (conglutins α , β , γ , and δ) between *L. albus* and *L. angustifolius*, which would

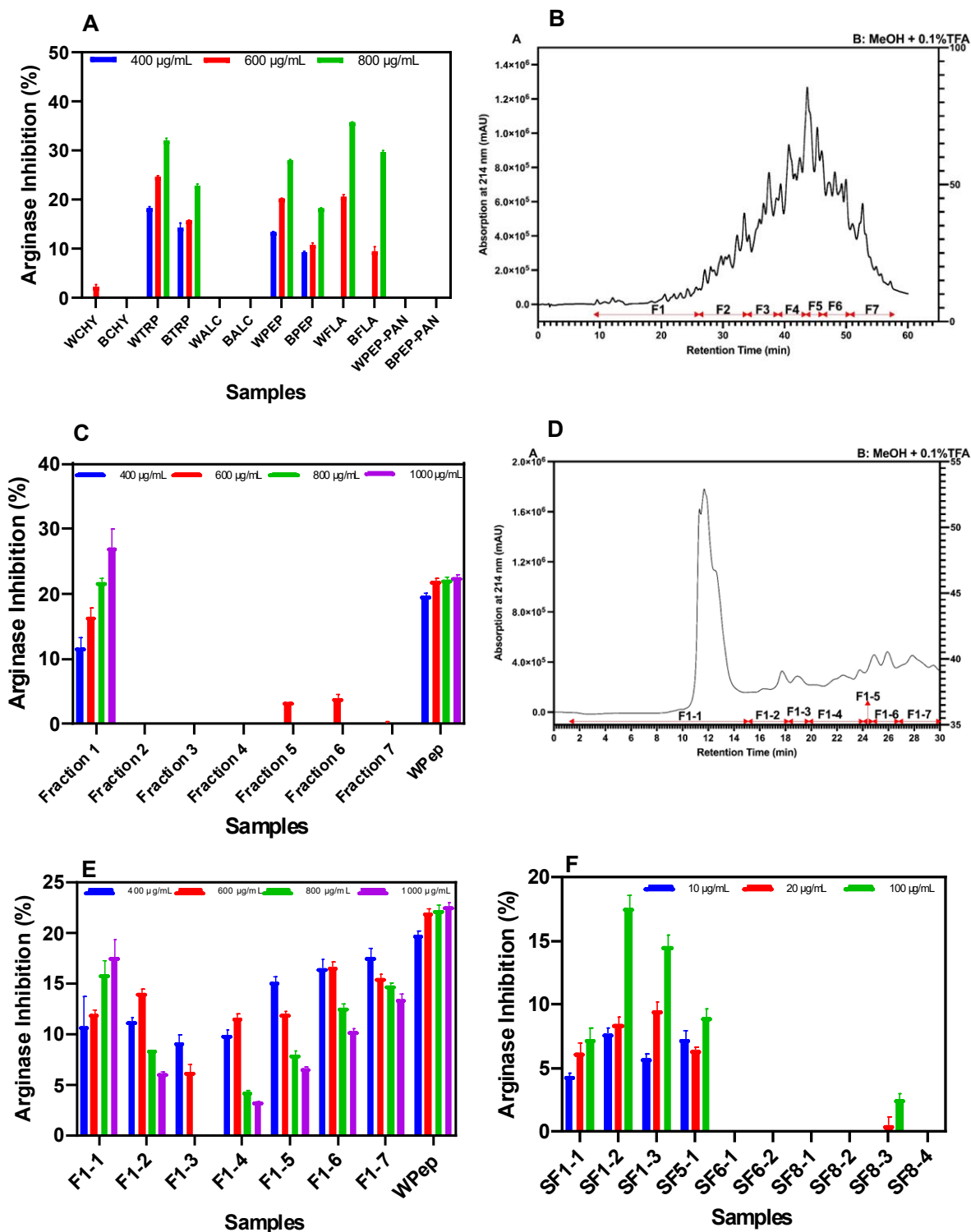


Figure 8.1 (A) Lupin protein hydrolysate arginase inhibition (B) First round of HPLC separated fractions (C) Arginase inhibitory activity of the first round of HPLC separated fractions (D) Second

round of HPLC separated fractions (E) Arginase inhibitory activity of the second round of HPLC separated fractions (F) Arginase inhibitory activities of synthesized peptide fractions.

Key: WCHY-White Chymotrypsin Hydrolysate, BCHY- Blue Chymotrypsin Hydrolysate, WTRP- White Trypsin Hydrolysate, BTRP- Blue Trypsin Hydrolysate, WALC-White Alcalase Hydrolysate, BALC- Blue Alcalase Hydrolysate, WPEP-White Pepsin Hydrolysate, BPEP- Blue Pepsin Hydrolysate, WPEP-PAN- White Pepsin Pancreatin Hydrolysate, BPEP-PAN- Blue Pepsin Pancreatin Hydrolysate. SF1-1 (KGPPSGSATPT), SF1-2 (DMGAVA), SF1-3 (VVALKKVGV LKKPGPT), SF5-1 (PPSVGY), SF6-1 (SGKVTST), SF6-2 (THGRSLVP), SF7-1 (VLAKGF), SF7-2 (LLGCPTYG), SF7-3 (RAKKLLVLRVGVPP), SF7-4 (HCLSFST).

influence the types of peptides released upon proteolysis (Chukwuejim et al., 2023) White lupin proteins have been reported to contain higher levels of basic amino acids, which may contribute to the generation of more potent arginase-inhibitory peptides (Chukwuejim & Aluko, 2025). The results suggest that white lupin proteins are a more suitable substrate for producing arginase-inhibitory peptides, with flavourzyme, trypsin, and pepsin representing the most effective proteases for this application.

8.3.2 HPLC Fractionation and Identification of Potent Arginase-Inhibitory Fractions

To isolate peptides with enhanced arginase inhibitory activity, the white lupin pepsin hydrolysate was subjected to two rounds of RP-HPLC fractionation. The first round of separation yielded seven fractions (F1–F7), with F1 demonstrating the highest arginase inhibitory activity (Figure 8.1B and 8.1C). This observation suggests that the most potent arginase-inhibitory peptides are relatively hydrophilic, as they eluted early in the reversed-phase gradient (Gosselin et al., 2024) This finding contrasts with an earlier report for pepsin hydrolysates of hemp seed protein, in which eight HPLC fractions were generated, and fraction F7 showed the strongest acetylcholinesterase inhibitory activity relative to the earlier-eluting fractions (F1–F6) (Malomo & Aluko, 2019) However, the early elution of the most active fraction is consistent with reports on other small arginase-inhibitory molecules, which require polar amino acid residues for optimal enzyme binding (Gosselin et al., 2024). The second round of HPLC separation of F1 produced seven sub-fractions (F1-1 through

F1-7), with F1-1, F1-5, F1-6, and F1-7 exhibiting the highest arginase inhibitory activities (Figure 8.1D and 8.1E). These sub-fractions were subsequently subjected to tandem mass spectrometry analysis, which identified ten distinct peptide sequences (Table 8.1). The identified peptides ranged from 6 to 16 amino acid residues in length, with average local confidence (ALC) scores of 55%-63%, supporting reliable sequence identification. The peptides exhibited diverse amino acid compositions, including sequences rich in hydrophobic residues (DMGAVA, VLAKGF), basic residues (VVALKKVGVLKKPGPT, RAKKLVLVRVGVPP), and proline (KGPPSGSATPT, PPSVGY). This structural diversity suggests that arginase inhibition may arise from multiple peptide–enzyme interaction modes involving hydrophobic, electrostatic, and conformational contributions.

8.3.3 Arginase Inhibitory Activity of Synthesized Peptides

The ten identified peptides were synthesized and evaluated for their arginase inhibitory activities (Figure 8.1F). Among these, SF1-2 (DMGAVA) and SF1-3 (VVALKKVGVLKKPGPT) demonstrated the highest arginase inhibitory activities and were selected for further kinetic and molecular studies. These two peptides represent structurally distinct classes of bioactive sequences: DMGAVA is a short hydrophobic hexapeptide, while VVALKKVGVLKKPGPT is a long polycationic 16-mer peptide containing four lysine residues. The contrasting structural features of these peptides provide valuable insights into the structure-activity relationships governing arginase inhibition. DMGAVA, with a molecular weight of approximately 562 Da, is composed predominantly of small hydrophobic residues (Ala, Val, Met) with a single acidic residue (Asp). The compact nature of this peptide likely enables it to access confined regions of the enzyme's binding pocket, while its hydrophobic character is likely to promote interactions with nonpolar residues in active site–proximal regions. Short hydrophobic peptides are generally

avored for therapeutic applications due to their enhanced membrane permeability, resistance to proteolytic degradation, and favorable pharmacokinetic properties (Nong & Hsu, 2021). The presence of the N-terminal aspartate may also contribute to binding through electrostatic interactions with positively charged residues on the enzyme surface.

In contrast, VVALKKVGVLKKPGPT represents a fundamentally different inhibitory scaffold. This polycationic peptide carries a net positive charge at physiological pH due to its four lysine residues, which can form multiple salt bridges and hydrogen bonds with negatively charged residues (Asp, Glu) in the arginase structure. The larger molecular size (~1,635 Da) enables this peptide to span a broader surface area on the enzyme, potentially engaging both the active site and peripheral regulatory regions simultaneously. The presence of the C-terminal proline-glycine-proline-threonine motif may confer conformational rigidity that optimizes the peptide's binding geometry. However, the high charge density and larger size of VVALKKVGVLKKPGPT may limit its cellular uptake and oral bioavailability compared to the more hydrophobic DMGAVA. The observation that two structurally distinct peptides exhibit effective arginase inhibition suggests that enzyme modulation may occur through multiple interaction modes, or that different molecular features can achieve inhibition through complementary mechanisms. This interpretation is supported by the competitive/mixed-type inhibition kinetics observed for both peptides, which are indicative of interactions with both substrate-binding regions and non-substrate binding interfaces. Similar behavior has been reported in other peptide–enzyme systems, in which peptides with varying physicochemical properties modulate enzyme activity through distinct yet convergent binding modes (Asen et al., 2022; Wang et al., 2025). Compared to previously reported arginase-inhibitory peptides, the lupin-derived peptides identified in this study represent a novel class of food-derived arginase modulators. Gao et al. (2021) reported that the synthetic peptide REGNH,

designed based on the C-terminal S-shaped motif of human arginase I, inhibited arginase I and II with IC₅₀ values of 2.4 ± 0.3 mM and 1.8 ± 0.1 mM, respectively, through a non-competitive mechanism. Interestingly, REGNH contains both charged (Arg, Glu) and polar (Asn, His) residues, representing an intermediate character between the hydrophobic DMGAVA and polycationic VVALKKVGVLKKPGPT identified in our study. The diversity of peptide structures that inhibit arginase suggests the enzyme's conformational flexibility and the potential for developing peptide-based therapeutics with nutraceutical relevance.

Table 8.1 Peptides Identified from MS for Sequencing

Fraction Position	Peptide	Average Local Confidence (ALC)
Fraction 1:287	KGPPSGSATPT (SF1-1)	59
Fraction 1:45	DMGAVA (SF1-2)	56
Fraction 1:439	VVALKKVGVLKKPGPT (SF1-3)	55
Fraction 5:223	PPSVGY (SF5-1)	61
Fraction 6:103	SGKVTSFT (SF6-1)	58
Fraction 6:213	THGRLSVP (SF6-2)	55
Fraction 7:283	VLAKGF (SF7-1)	63
Fraction 7:233	LLGCPTYG (SF7-2)	56
Fraction 7:256	RAKKLVLRVGVPP (SF7-3)	56
Fraction 7:336	HCLSFST (SF7-4)	56

8.3.4 Kinetics of Arginase Inhibition

Enzyme inhibition kinetics is useful for understanding the mechanism by which inhibitors interact with their target enzymes. Lineweaver-Burk plots (Figure 8.2) were constructed to determine the kinetic parameters and mode of inhibition for DMGAVA and VVALKKVGVLKKPGPT. The kinetic parameters presented in Table 8.2 reveal distinct inhibition patterns for the two peptides, reflecting their different structural characteristics. For the short hydrophobic peptide DMGAVA, the apparent Michaelis constant (K_m') increased progressively from 36.74 mM (control) to 50.53 mM (100 μ M peptide), representing a 37.5% increase at the highest inhibitor concentration. Concurrently, the maximum velocity (V_{max}) decreased slightly by about 15%. This kinetic profile is characteristic of mixed-type inhibition, where the inhibitor affects both substrate binding (increased K_m) and catalytic turnover (decreased V_{max}). The calculated inhibition constant (K_i) of approximately 266 μ M indicates moderate-to-strong binding affinity to arginase. The effects on both K_m and V_{max} suggest that DMGAVA binds to both the free enzyme and the enzyme-substrate complex. For the long polycationic peptide VVALKKVGVLKKPGPT, a different kinetic pattern emerged. The K_m' increased modestly from 36.74 mM (control) to 42.98 mM (100 μ M), representing only a 17.0% increase at the highest concentration, less than half the effect observed with DMGAVA. This kinetic profile indicates a predominantly competitive component of inhibition, in which the peptide primarily interferes with substrate binding rather than with catalytic efficiency. The calculated K_i of approximately 589 μ M is higher than that of DMGAVA, indicating weaker overall binding affinity.

The contrasting kinetic behaviors of these peptides can be rationalized by their structural differences. The compact hydrophobic DMGAVA can engage residues within the active-site cleft and induce conformational changes that affect substrate binding and catalysis, thereby altering K_m and V_{max} . In contrast, the large polycationic VVALKKVGVLKKPGPT may bind primarily at

the entrance of the active site or at peripheral anionic regions, sterically hindering substrate access (competitive component) without substantially altering the intrinsic catalytic machinery. The multiple positive charges on VVALKKVGVLKKPGPT may favor interactions with the negatively charged residues (D, E) lining the substrate channel, thereby competing with the positively charged guanidinium group of L-arginine for electrostatic interactions. The mixed-type inhibition pattern observed for DMGAVA is consistent with previous reports on peptide-based enzyme inhibitors. Gao et al. (2021) reported non-competitive inhibition of arginase by the synthetic peptide REGNH, indicating allosteric modulation. Similarly, Asen et al. (2022) demonstrated that pea protein-derived peptides exhibited mixed-type inhibition against acetylcholinesterase. The mechanistic diversity observed among arginase-inhibitory peptides suggests that multiple strategies exist for modulating enzyme activity, which may be exploited for therapeutic development.

Table 8.2 Kinetic Parameters for the Inhibition of Arginase Activity by SF1-2 and SF1-3 Peptides

	Control	20 μ M	100 μ M
SF1-2 (DMGAVA)			
K_m or K'_m (mM)	36.74	40.59	50.53
V_{max} or V'_{max}	0.64	0.57	0.54
K_i (μM)		~266 μ M	
SF1-3 (VVALKKVGVLKKPGPT)	Control	20 μ M	100 μ M
K_m or K'_m (mM)	36.74	37.10	42.98
V_{max} or V'_{max}	0.64	0.64	0.60
K_i (μM)		~589 μ M	

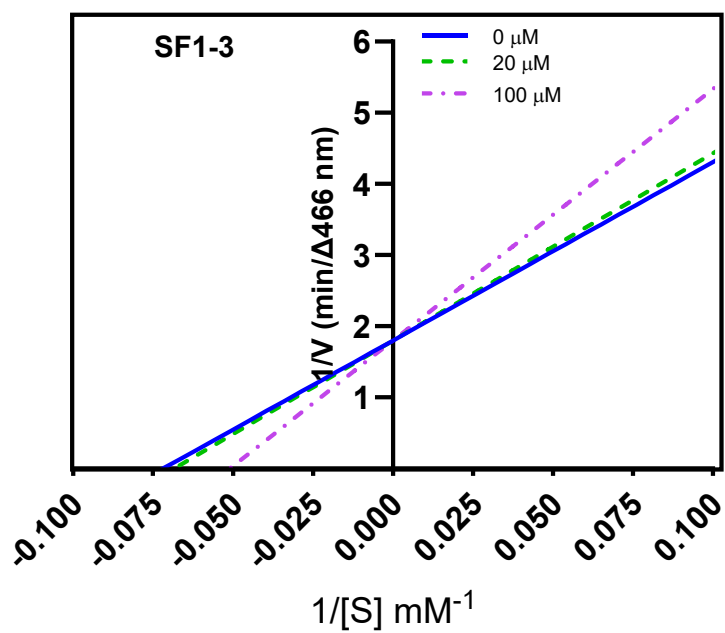
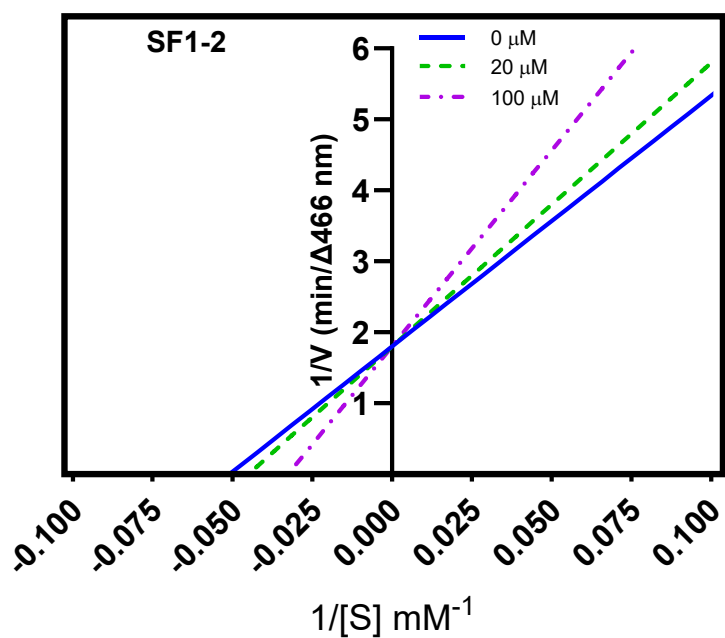


Figure 8.2 Kinetics of arginase inhibition (Lineweaver-Burk plot) of the synthesized peptides (A). SF1-2 (DMGAVA) (B). SF1-3 (VVALKKVGV LKKPGPT).

8.3.5 Molecular Docking Analysis

Molecular docking simulations were performed to elucidate the binding interactions between the lupin peptides and human arginase I (PDB: 2AEB). The docking scores, binding free energies (ΔG), dissociation constants (K_d), and interacting residues are summarized in Table 8.3. VVALKKVGVLKKPGPT exhibited a more favorable docking score (-166.719) compared to DMGAVA (-113.443), reflecting the greater number of atomic contacts possible with the larger peptide. However, both peptides displayed similar binding free energies (ΔG of -8.4 and -8.3 kcal/mol, respectively) and dissociation constants (K_d of 7.4×10^{-7} M and 8.4×10^{-7} M), indicating comparable thermodynamic stability of the peptide-enzyme complexes at equilibrium.

The molecular docking analysis revealed distinct binding patterns for the two peptides that correlate with their structural characteristics and kinetic behaviors (Figure 8.3A and 8.3B). The short hydrophobic peptide DMGAVA primarily interacted with arginase via an extensive hydrogen-bonding network involving ARG21, ASP128, ILE129, ASN130, ASP181, ASP183, and THR246, with additional hydrophobic interactions with PRO20. Notably, ASP128 and ASN130 are located in the S2 substrate-binding loop, while ASP181 and ASP183 are positioned near the binuclear manganese cluster that constitutes the catalytic center of arginase. The direct engagement of these catalytically critical residues provides a molecular rationale consistent with the observed reduction in V_{max} observed in kinetics studies. Furthermore, THR246 is involved in substrate orientation within the active site, and its interaction with DMGAVA would contribute to the observed increase in K_m by interfering with optimal substrate positioning.

In contrast, VVALKKVGVLKKPGPT exhibited a more extensive but peripheral network of interactions. Hydrogen bonds were formed with GLU151, ASP181, VAL182, TYR188, LYS191, TYR197, and ARG205, while hydrophobic contacts involved THR131, LEU152, LYS155, VAL182, PRO184, TYR188, ILE189, and THR192. Many of these residues (GLU151, LEU152,

TYR188, TYR197) are located at the rim of the active site entrance rather than within the catalytic pocket. This peripheral binding mode is consistent with the predominantly competitive kinetic pattern observed for VVALKKVGVLKKPGPT, where the peptide sterically hinders substrate access without substantially perturbing the catalytic machinery. The interaction with ASP181, which is common to both peptides, represents a critical anchoring point that may be essential for arginase inhibition regardless of peptide structure. The docking results provide a molecular rationale for the kinetic observations. The ability of DMGAVA to penetrate the active site and directly engage catalytic residues (ASP181, ASP183, THR246) accounts for its mixed-type inhibition, with substantial effects on both K_m and V_{max} . Conversely, the peripheral binding mode of VVALKKVGVLKKPGPT, with limited access to the catalytic center, accounts for its predominantly competitive behavior with modest effects on catalytic efficiency.

Table 8.3 Peptide-Arginase Molecular Docking

SN	Peptides	Human arginase I (PDB: 2AEB)			
		HPEPDOCK	PRODIGY		Enzyme Interacting Residue
		DOCK SCORE (TOP MODEL)	ΔG (kcal mol ⁻¹)	K _d (M) at 25°C	
1	SF1-2 (DMGAVA)	-113.443	-8.3	8.4e-07	Hydrophobic interaction: PRO20 Hydrogen bonds: ARG21, ASP128, ILE129, ASN130, ASP181, ASP183, THR246
2	SF1-3 (VVALKKVGV LKKPGPT)	-166.719	-8.4	7.4e-07	Hydrophobic interaction: THR131, LEU152, LYS155, VAL182, PRO184, TYR188, ILE189, THR192 Hydrogen bonds: GLU151, ASP181, VAL182, TYR188, LYS191, TYR197, ARG205

Table 8.4 MMGBSA Calculated Binding Free Energies for Full 100ns Peptide-Enzyme Complex System*

SN	Complex	MMGBSA ΔG (kcal.mol ⁻¹)						
		$\Delta VDWAAALS$	ΔEEL	ΔEGB	$\Delta ESURF$	$\Delta GGAS$	$\Delta GSOLV$	$\Delta TOTAL$
1	SF1-2 (DMGAVA) - Arginase I	-17.96±6.50	-83.26±1.40	70.09±21.60	-4.36±0.64	-101.23±28.78	65.73±21.76	-35.49±9.42
2	SF1-3 (VVALKKVGVLLKKPGPT)-Arginase I	-41.84±11.49	-558.25± 96.67	568.20±91.50	-7.75±1.91	-600.09±101.24	560.45±90.37	-39.64±14.11

*MMGBSA, Molecular Mechanics-Generalized Born Surface Area. Data presented in Mean $\pm$ Standard deviation (SD). $\Delta VDWAAALS$: van der Waals energy. ΔEEL : Electrostatic energy. ΔEGB : Polar solvation energy. $\Delta ESURF$: Nonpolar solvation energy. $\Delta GGAS$: Gas-phase energy. $\Delta GSOLV$: solvation free energy. $\Delta TOTAL$: Total binding energy estimate. $\Delta GSOLV = \Delta EGB + \Delta ESURF$. $\Delta TOTAL = \Delta GGAS + \Delta GSOLV$.

8.3.6 Molecular Dynamics (MD) Simulations

MD simulations were performed for 100 ns to assess the conformational stability, binding persistence, and dynamic behavior of the peptide-arginase complexes. The radius of gyration (R_g), root mean square deviation (RMSD), and root mean square fluctuation (RMSF) were analyzed to evaluate the structural compactness, overall stability, and residue-specific flexibility of the complexes, respectively. The R_g profiles for both peptide-enzyme complexes (Figure 8.3C and 8.3D) provide insight into the overall compactness of the protein structure upon ligand binding (Ulmschneider & Ulmschneider, 2018). For the DMGAVA-arginase complex, the R_g values remained stable throughout the 100 ns trajectory, fluctuating within a narrow range, indicating that the enzyme maintains its native fold upon binding the short hydrophobic peptide. Similarly, the VVALKKVGVLKKPGPT-arginase complex exhibited consistent R_g values, suggesting that despite the larger size of this peptide, its binding does not induce significant expansion or collapse of the enzyme structure. The comparable R_g profiles for both complexes indicate that neither peptide causes substantial global structural perturbation, consistent with reversible inhibition rather than enzyme denaturation. The maintenance of structural compactness is functionally relevant, as it suggests that the inhibited enzyme retains the potential to recover activity upon inhibitor dissociation.

The RMSD profiles (Figure 8.4A and 8.4B) revealed distinct equilibration behaviors for the two peptide-enzyme complexes. The DMGAVA-arginase complex reached conformational equilibrium rapidly within the first 15-20 ns of simulation, with subsequent RMSD values stabilizing at approximately 0.15-0.20 nm. This rapid equilibration is consistent with stable, well-structured binding between the compact hydrophobic peptide and its binding site within the active-site cleft. The low RMSD plateau indicates that the complex adopts a stable conformation with

minimal structural drift over the simulation timescale. In contrast, the VVALKKVGVLKKPGPT-arginase complex exhibited a longer equilibration phase, requiring approximately 30-40 ns to reach conformational stability, with RMSD values fluctuating in a slightly higher range (0.20-0.25 nm). The extended equilibration period can be attributed to the larger conformational space accessible to the 16-mer peptide and the need to optimize multiple electrostatic interactions between its four lysine residues and complementary enzyme residues. The higher RMSD plateau reflects the inherent flexibility of the long peptide chain and the dynamic nature of peripheral binding interactions. Importantly, both complexes reached stable plateaus within the simulation timeframe, confirming that the docking-derived binding poses are energetically favorable and persist over biologically relevant timescales.

The RMSF analysis (Figure 8.4C and 8.4D) revealed residue-specific flexibility patterns that show the dynamic consequences of peptide binding. For the DMGAVA-arginase complex, reduced RMSF values were observed for residues in the active site region, particularly those identified as interaction partners in the docking analysis (ASP128-ASN130, ASP181-ASP183, THR246). This localized rigidification indicates that DMGAVA binding stabilizes the active site architecture, which may contribute to inhibition by restricting the conformational flexibility required for substrate binding and catalysis. Loop regions distant from the binding site maintained their native flexibility, consistent with the localized binding mode of this compact peptide. For the VVALKKVGVLKKPGPT-arginase complex, the RMSF profile showed a broader pattern of altered dynamics. Reduced flexibility was observed not only at the active-site entrance but also in peripheral regions involved in peptide binding. Interestingly, some active site residues retained higher flexibility compared to the DMGAVA complex, consistent with the docking observation that VVALKKVGVLKKPGPT binds peripherally without deeply penetrating the catalytic pocket.

This differential flexibility pattern supports the kinetic observation that VVALKKVGVLKKPGPT primarily affects substrate access rather than catalytic efficiency. Overall, the MD simulations confirm that both lupin-derived peptides form stable complexes with arginase I over 100 ns trajectories. The distinct dynamic signatures localized active-site stabilization for DMGAVA versus broader peripheral interaction patterns for VVALKKVGVLKKPGPT—are consistent with their differing kinetic behaviors and highlight complementary mechanisms by which structurally diverse food-derived peptides can modulate arginase activity.

8.3.7 Binding Free Energy Analysis: Molecular Mechanics-Generalized Born Surface Area (MMGBSA)

The binding free energy for the peptide-arginase complexes was calculated using the MMGBSA method over the full 100 ns simulation trajectory (Table 8.4). The total binding free energies (ΔTOTAL) were -35.49 ± 9.42 kcal/mol for the DMGAVA-arginase complex and -39.64 ± 14.11 kcal/mol for the VVALKKVGVLKKPGPT-arginase complex. The negative binding free energies for both complexes indicate that the interactions between the peptides and arginase I are thermodynamically favorable. Analysis of the individual energy components revealed fundamentally different binding thermodynamics for each peptide, reflecting their contrasting structural characteristics. For the short hydrophobic peptide DMGAVA, the binding energetics showed a balanced profile: van der Waals interactions contributed -17.96 ± 6.50 kcal/mol, while electrostatic interactions provided -83.26 ± 1.40 kcal/mol. The polar solvation penalty ($\Delta\text{EGB} = 70.09 \pm 21.60$ kcal/mol) was relatively modest, and the nonpolar solvation term ($\Delta\text{ESURF} = -4.36 \pm 0.64$ kcal/mol) contributed favorably through burial of hydrophobic surface area. The gas-phase binding energy ($\Delta\text{GGAS} = -101.23 \pm 28.78$ kcal/mol) was substantially offset by the solvation penalty ($\Delta\text{GSOLV} = 65.73 \pm 21.76$ kcal/mol), yielding the net favorable binding energy. This

thermodynamic profile is characteristic of binding interactions dominated by shape complementarity and moderate electrostatic contributions, consistent with the mixed hydrophobic/polar nature of DMGAVA.

For the peptide VVALKKVGVLKKPGPT, the energy decomposition revealed a markedly different binding mechanism. The electrostatic contribution was exceptionally large ($\Delta EEL = -558.25 \pm 96.67$ kcal/mol), reflecting the formation of multiple salt bridges between the four lysine ϵ -amino groups and negatively charged enzyme residues (Asp, Glu). The van der Waals contribution was also substantial ($\Delta VDWALS = -41.84 \pm 11.49$ kcal/mol), consistent with the extensive contact surface between this large peptide and the enzyme. However, these highly favorable gas-phase interactions ($\Delta GGAS = -600.09 \pm 101.24$ kcal/mol) were largely offset by an enormous polar solvation penalty ($\Delta EGB = 568.20 \pm 91.50$ kcal/mol), which represents the energetic cost of desolvating the highly charged lysine residues upon complex formation. The nonpolar solvation term ($\Delta ESURF = -7.75 \pm 1.91$ kcal/mol) was more favorable than for DMGAVA, reflecting the burial of greater hydrophobic surface area.

The comparable total binding free energies for both peptides (-35.49 vs -39.64 kcal/mol), despite their vastly different energy component profiles, illustrate a fundamental principle in molecular recognition: thermodynamic equivalence can be achieved through diverse binding mechanisms. DMGAVA achieves favorable binding through efficient shape complementarity and moderate electrostatic interactions with minimal desolvation penalty, while VVALKKVGVLKKPGPT relies on extensive electrostatic complementarity that is substantially offset by desolvation costs. This phenomenon, known as enthalpy-entropy compensation, is commonly observed in protein-ligand interactions and explains how structurally diverse molecules can exhibit similar binding affinities (Chodera & Mobley, 2013). The higher standard deviation observed for the binding free

energy of VVALKKVGVLKKPGPT likely reflects the greater conformational flexibility of this longer peptide and the dynamic nature of its electrostatic interactions, which may transiently form and dissociate over the course of the simulation. This interpretation is consistent with the longer RMSD equilibration time and the higher RMSF values observed for this complex in the MD analysis.

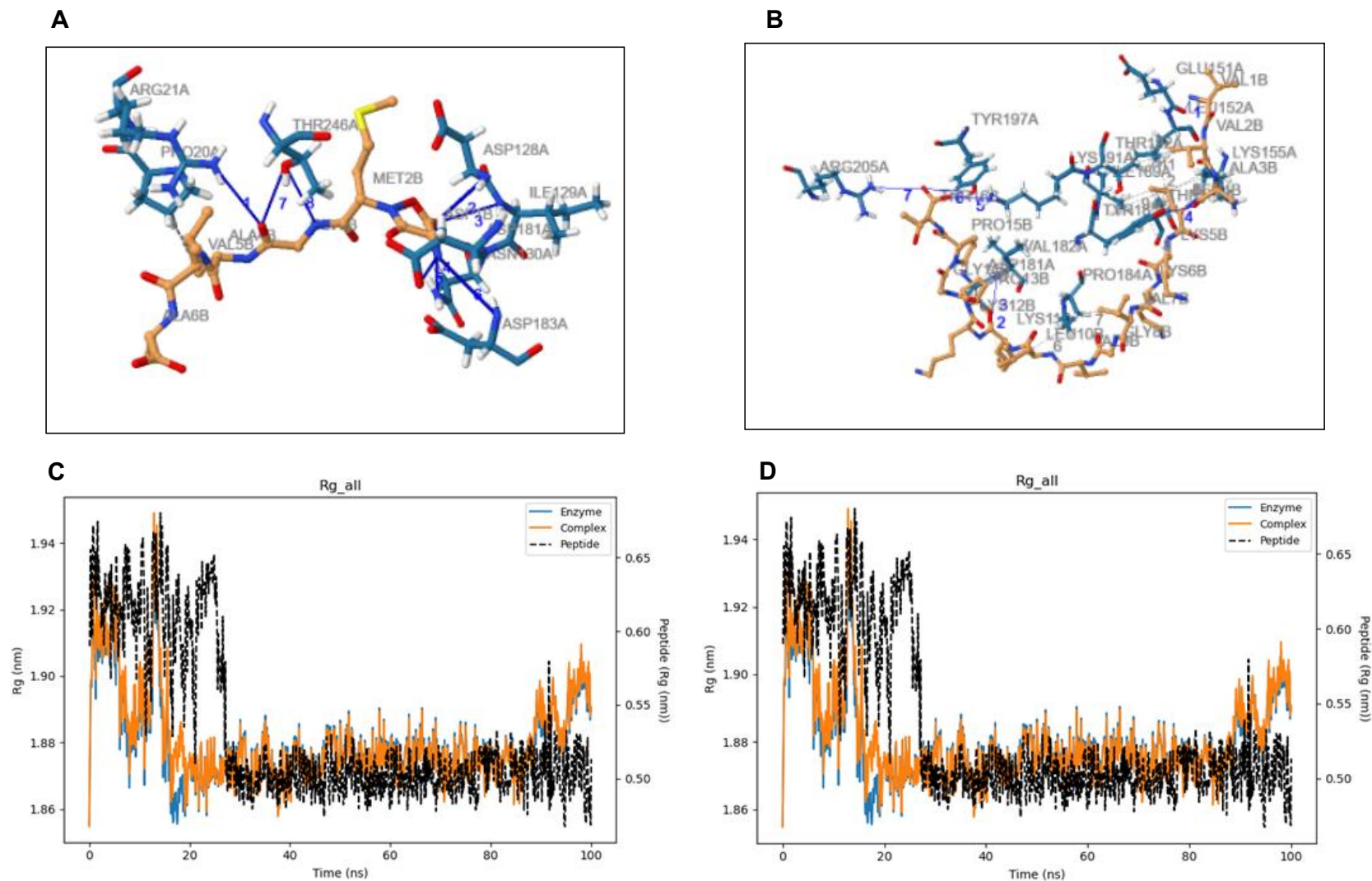


Figure 8.3 Molecular docking binding interactions of A. SP1-2 (DMGAVA) and B. SP1-3 (VVALKKVGV LKKPGPT). Radius of gyration (Rg) of C. SP1-2 (DMGAVA) and D. SP1-3 (VVALKKVGV LKKPGPT).

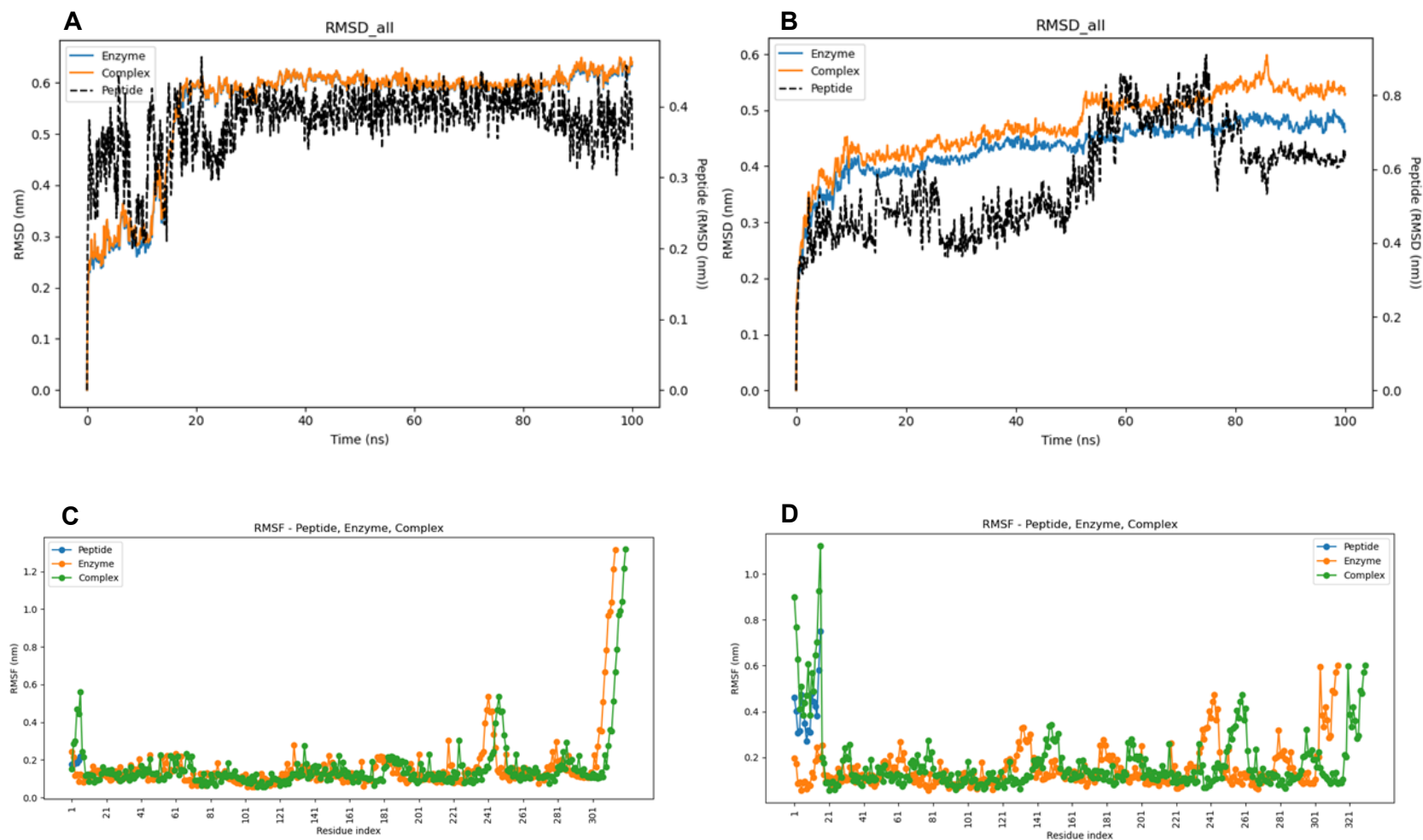


Figure 8.4 Root mean square deviation of A. SP1-2 (DMGAVA) and B. SP1-3 (VVALKKVGVLKKPGPT). Root mean square fluctuation of C. SP1-2 (DMGAVA) and D. SP1-3 (VVALKKVGVLKKPGPT).

8.4 CONCLUSION

This study demonstrates that lupin seed proteins are a previously unexplored source of arginase-inhibitory peptides and that enzymatic hydrolysis can generate peptides with distinct arginase-inhibitory mechanisms. White lupin consistently exhibited more potent arginase inhibitory activity than blue lupin, highlighting species-specific differences in protein composition that influence the release of bioactive peptides. The identification of both a short hydrophobic peptide and a longer polycationic peptide with comparable inhibitory activity reveals that structurally diverse food-derived peptides can modulate arginase activity through complementary molecular strategies. These findings highlight the conformational adaptability of arginase and suggest that inhibition is not restricted to a single binding mode or peptide architecture. The integration of kinetic analysis with molecular modeling further supports the concept that peptide size, charge distribution, and flexibility collectively govern enzyme modulation. The ability to generate distinct arginase-modulating peptides from lupin proteins expands their functional relevance beyond conventional nutritional applications. This study provides a molecular framework for the rational design of lupin-based functional ingredients targeting arginase-related metabolic pathways. Future investigations should focus on peptide stability during gastrointestinal digestion, bioaccessibility, and *in vivo* physiological relevance to support translation into functional food systems.

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AUTHOR CONTRIBUTIONS

Stanley Chukwuejim, Writing – original draft, Investigation, Methodology, Formal analysis; Timilehin D. Oluwajuyitan, Investigation, Methodology, Formal analysis, Manuscript review and editing; Deepak Kadam, Methodology, Formal analysis, Manuscript review and editing. Toluwase H. Fatoki: Methodology, Formal analysis, Manuscript review and editing; Rotimi E. Aluko, Conceptualization, Validation, Supervision, Project administration, Investigation, Funding acquisition, Manuscript review and editing.

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TRANSITION STATEMENT

Chapter 8 (Manuscript 7) established that lupin seed proteins can serve as a source of arginase-inhibitory peptides. Enzymatic hydrolysis with multiple proteases followed by two rounds of reverse-phase HPLC fractionation and tandem mass spectrometry identified multiple peptide sequences, including ten from the most active fractions. Two structurally distinct peptides—the short hydrophobic hexapeptide DMGAVA and the long polycationic 16-mer VVALKKVGVLKKPGPT—demonstrated the highest arginase inhibition and were shown to operate through complementary mixed-type inhibitory mechanisms. Molecular docking and 100 ns molecular dynamics simulations revealed that DMGAVA accesses the active-site cavity directly, while VVALKKVGVLKKPGPT engages an extended binding surface involving both active-site and peripheral residues. These findings provided a molecular framework for understanding how food-derived peptides of varying size and physicochemical character can modulate arginase activity. While Chapter 8 employed a classical experimental discovery approach—hydrolysis, chromatographic purification, sequencing, and kinetic characterization—this approach is resource-intensive and limited to peptides released under the specific digestion conditions tested. To complement and extend these findings, Chapter 9 (Manuscript 8) adopts a rational, computation-first strategy. Starting from the REGNH peptide scaffold and mining the white lupin genome for C-terminal arginine-containing sequences, Chapter 9 integrates *in silico* proteolysis, ADME and allergenicity predictions, target prediction, molecular docking, and molecular dynamics simulations to identify and rank candidate arginase-inhibitory peptides, followed by the synthesis of a focused subset for experimental validation. This integrated computational–experimental approach not only identifies new arginase-inhibitory peptides (notably the tripeptide DQR) but also critically evaluates the predictive accuracy of current *in silico* methods, providing

insights into the discrepancies between computational binding affinity and actual *in vitro* inhibitory function that are essential for future peptide optimization efforts.

CHAPTER NINE

MANUSCRIPT 8

***IN SILICO* RATIONAL INVESTIGATION OF C-TERMINAL ARGININE-CONTAINING WHITE LUPIN (*LUPINUS ALBUS*) PROTEIN-DERIVED PEPTIDES AS ARGINASE INHIBITORS**

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ABSTRACT

Arginase is a binuclear manganese metalloenzyme that modulates L-arginine availability and downstream metabolic pathways. It was hypothesized that C-terminal arginine-containing peptides generated through simulated gastrointestinal digestion of white lupin (*Lupinus albus*) proteins would exhibit arginase inhibitory activity. To test this hypothesis, integrated computational and experimental approaches were employed to identify and characterize arginase-inhibitory peptides from white lupin proteins. Based on the REGNH peptide scaffold, 29 derivative sequences were designed and used to screen the lupin genome, followed by *in silico* proteolysis, yielding 24 C-terminal arginine-containing peptides. None of the peptides showed evidence of allergenicity. Target prediction identified DR, CR, ER, and QR as top candidates, while molecular docking and 100 ns molecular dynamics simulations revealed isoform-specific binding preferences and stability patterns. Five peptides (CQR, DR, DQR, QER, REGNH) were synthesized and evaluated against arginase I using an *in vitro* colorimetric assay. DQR showed the highest inhibitory activity among the tested peptides, achieving approximately 17% inhibition at 100 µg/mL. Kinetic analysis indicated an apparent mixed-type inhibition pattern, with increased K_m (31.2 to 35.4 mM) and decreased V_{max} (0.72 to 0.70 $\Delta\text{Abs}/\text{min}$). Despite a top computational ranking, DR showed negligible *in vitro* activity (<2%), highlighting the limitations of binding-affinity predictions for forecasting functional enzyme inhibition. These findings demonstrate the potential of integrated computational-experimental approaches for identifying arginase-modulatory peptides encrypted within food proteins

Keywords: Bioactive peptides, Virtual screening, Nutraceuticals, Enzyme inhibition kinetics, Structure-activity relationship, Peptide design

9.1 INTRODUCTION

Food-derived bioactive peptides (BAPs), typically 2–20 amino acids in length, are encrypted within parent food proteins and released upon enzymatic hydrolysis or gastrointestinal digestion (Udenigwe & Aluko, 2012). *In silico* approaches have become increasingly valuable for accelerating the discovery of food-derived BAPs. For example, virtual proteolysis using the BIOPEP-UWM database combined with molecular docking has been successfully employed to identify ACE-inhibitory peptides from sheep milk proteins (Iram et al., 2022) and DPP-IV inhibitory peptides from discarded eggshell membrane (Zhu et al., 2024). Similarly, integrated computational pipelines involving *in silico* hydrolysis, ADME screening, and molecular docking have identified antioxidant peptides from lupin hydrolysates (Guo et al., 2018), ACE-inhibitory peptides from Bambara groundnut (Saetang et al., 2025), and renin-inhibitory peptides from Tartary buckwheat albumin hydrolysates (Li et al., 2024). These studies demonstrate that *in silico*-guided approaches provide an efficient framework for identifying bioactive peptide candidates from food proteins prior to experimental validation.

Arginase is a binuclear manganese metalloenzyme that catalyzes the hydrolysis of L-arginine to L-ornithine and urea, playing a central role in nitrogen metabolism and the regulation of L-arginine availability for downstream metabolic pathways including nitric oxide synthesis (Cama et al., 2003; Canè et al., 2025). While synthetic arginase inhibitors have been extensively studied (Pudlo et al., 2017), food-derived peptides with arginase-modulatory activity have not yet been reported. To date, only one peptide-based arginase inhibitor (REGNH) has been described in the literature (Gao et al., 2021). Notably, peptides containing C-terminal arginine residues have shown enhanced bioactivity due to their positive charge and capacity for electrostatic interactions with biological targets (Gautam et al., 2015; Zhao et al., 2020) and have demonstrated inhibitory activities against

acetylcholinesterase and butyrylcholinesterase (Asen et al., 2023), supporting the functional significance of this structural feature in food-derived peptides.

White lupin (*Lupinus albus*), a legume of the Fabaceae family containing 35–40% protein by dry weight, is an established source of bioactive peptides (Chukwuejim et al., 2024). Lupin protein hydrolysates exhibit antioxidant and ACE-inhibitory activities (Guo et al., 2018), and peptides from lupin have demonstrated DPP-IV inhibitory properties (Cruz-Chamorro et al., 2023; Santos-Sánchez et al., 2022). The high arginine content of lupin proteins, combined with their established track record as sources of enzyme-inhibitory peptides, makes white lupin a rational candidate for exploring arginase-modulatory peptides. However, the potential of lupin-derived peptides to modulate arginase activity has not been investigated, and the structural determinants governing peptide-arginase interactions in food-derived peptides remain unknown.

The present study addresses this gap by employing an integrated *in silico* and *in vitro* approach to identify and characterize arginase-inhibitory peptides from white lupin proteins. It was hypothesized that simulated gastrointestinal digestion of white lupin proteins would release C-terminal arginine-containing peptides with arginase inhibitory activity. To test this, peptide motifs based on the REGNH sequence (Gao et al., 2021) — the only reported peptide-based arginase inhibitor — were used to guide proteome screening, followed by *in silico* enzymatic hydrolysis, ADME and allergenicity prediction, target prediction, molecular docking, and molecular dynamics simulations. Selected candidate peptides were then synthesized and experimentally evaluated for arginase I inhibitory activity and kinetic characterization. This approach aims to elucidate the structure-activity relationships governing peptide-arginase interactions in food protein-derived peptides.

9.2 MATERIALS AND METHODS

9.2.1 Arginase compound assembling

The small organic compounds that bind to arginase I and II were obtained from the DrugBank database (www.drugbank.ca; accessed: 20-11-2024). A synthetic peptide (sequence: REGNH) that binds to arginase I and II was also obtained from the literature (Gao et al., 2021).

9.2.2 Design of synthetic peptide derivatives and screening of white lupin genome proteins

The white lupin genome proteins file was obtained in FASTA format from https://www.whitelupin.fr/genome_files/prot.fasta (accessed: 05-07-2024). A synthetic peptide (sequence: REGNH) was used for the rational design of 29 sequence derivatives (See Supplementary File 1, Table S1), which were then used to screen the white lupin genome protein file. The white lupin proteins that contain any of these 29 derivatives were selected (See Supplementary File 2). and used for further analyses.

9.2.3 *In silico* protein hydrolysis

The selected white lupin proteins sequences obtained in step 2.2 were subjected to simulated enzymatic hydrolysis using a combination of digestive enzymes, namely pepsin (EC 3.4.23.1), chymotrypsin (EC 3.4.21.1), and trypsin (EC 3.4.21.4) concurrently, utilizing the BIOPEP-UWM webserver (<https://biochemia.uwm.edu.pl/en/biopep-uwm-2/>; accessed: 25-11-2024) (See Supplementary File 3). The hydrolysate peptides thus obtained were subsequently subjected to screening with pancreatic elastase (EC 3.4.21.36) digestion (Minkiewicz et al., 2019). The cleavage sites of the enzymes are provided in supplementary file 1 (Table S2). *In silico* digestion assumed physiological pH (pH 7.4), the effect of varied pH and time were not considered. Among the resulting hydrolysate peptide sequences, those with C-terminal arginine residues were chosen for further analysis. The filtering criteria was based on the susceptibility of the sequence to the proteases cleavage, and prior understanding from the organic compounds that are arginase

inhibitors (method 2.1) should contain arginine-like molecule. These selected sequences were then converted to the Simplified Molecular Input Line Entry Specification (SMILES) format using the BIOPEP-UWM webserver, as previously described (Fatoki et al., 2023).

9.2.4 *In silico* pharmacokinetic and allergenicity prediction

The assessment of *in silico* pharmacokinetic or ADME (absorption, distribution, metabolism, and excretion) screening for the chosen hydrolysate peptides containing C-terminal arginine, as well as for standard compounds, was conducted on the SwissADME webserver, accessible at www.swissadme.ch, accessed: 27-11-2024 (Daina et al., 2017). This analysis was carried out using the SMILES format of the peptides. Additionally, the evaluation of allergenicity was performed using the AllerCatPro 2.0 webserver, accessible at <http://allercatpro.bii.a-star.edu.sg>, accessed: 27-11-2024 (Nguyen et al., 2022).

9.2.5 *In silico* arginase target prediction

The SMILES representation of each of the chosen hydrolysate peptides containing C-terminal arginine, obtained through the *in silico* enzymatic hydrolysis, as well as the SMILES of the standard compounds, were employed for target prediction analysis on the SEA Search webserver, accessible at <https://sea.bkslab.org/>, accessed: 28-11-2024 (Keiser et al., 2007). During this analysis, targets that included human arginase I and II were identified.

9.2.6 Molecular docking studies

Peptide-protein molecular docking was conducted using AutoDock Vina v1.2.3, as detailed in the work by Trott & Olson, (2010) and Eberhardt et al., (2021). Blind docking approach were used and executed in accordance with previous studies (Fatoki et al., 2023; Obeme-Nmom et al., 2023), in order to capture the types of inhibition exhibited by these peptides on binding to the arginase sites (active site or allosteric site). The small organic compounds (ligands) obtained in step 2.1 as

well as REGNH peptide, were used to validate and compare our docking. Human arginase I and II were obtained from the protein data bank (PDB ID: 2AEB and 4IXU). Docking grid parameter used for human arginase I (Center: 69.48, 24.449, 34.841. Size: 126, 126, 126. Spacing: 0.375) and human arginase II (Center: 37.769, 87.718, 57.837. Size: 126, 126, 126. Spacing: 0.375) respectively ensure full coverage of the structure. Before initiating docking, the peptides, ligands and human arginases were prepared in pdbqt format utilizing AutoDock Tools (ADT) v1.5.6, developed by Morris et al., (2009). Subsequently, the binding affinity was determined, and the close interactions between the ligands and the target proteins were scrutinized and visualized using ezLigPlot on the ezCADD webserver available at <http://dxulab.org/software> accessed: 30-11-2024 (Tao et al., 2019).

9.2.7 Molecular dynamics simulation

Molecular dynamics (MD) simulations of human arginase I and II in complex with four of our peptides (CQR, DR, DQR and QER), one organic compound (DB03144) and a synthetic peptide REGNH, were investigated. Desmond module in Schrödinger molecular modeling suite v2020-3 was used to implement the complex simulation for 100 ns (Bowers et al., 2006; Pirolli et al., 2023). The protein-peptide complexes at the initial stage for these simulations were acquired from the docking studies. The preparation of the protein-peptide complexes involved maestro's protein preparation wizard, which encompassed optimization and minimization steps of the complexes. All systems were prepared utilizing the System Builder tool. For the solvent model, an orthorhombic box was chosen, employing the TIP3P (Transferable Intermolecular Interaction Potential 3 Points) model. During the simulations, the OPLS-2005 force field was employed (Shivakumar et al., 2010). To achieve neutral systems, counter ions (0.15 M NaCl) were introduced to replicate physiological conditions, as described by Fatoki (2023a). The NPT ensemble, which

conserves moles (N), pressure (P), and temperature (T), was chosen for the simulations, maintaining a temperature of 300 K and a pressure of 1 atm. Prior to the commencement of the simulations, the models were allowed to relax. Throughout the simulations, trajectory data were saved every 100 ps, and post-simulation analysis of these trajectories was conducted, involving the determination of various parameters, including root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), protein-ligand interaction profile, radius of gyration (Rg), and solvent accessibility surface area (SASA). Furthermore, prime molecular mechanics/generalized Born surface area (MMGBSA) calculations were performed, enabling the assessment of binding free energy. The binding free energy calculation procedure was as follows:

- MMGBSA $\Delta G^{\text{bind}} = \Delta G^{\text{complex}} - \Delta G^{\text{protein}} - \Delta G^{\text{ligand}}$
- MMGBSA $\Delta G^{\text{bind}} = \Delta G^{\text{Coulomb}} + \Delta G^{\text{Covalent}} + \Delta G^{\text{Hbond}} + \Delta G^{\text{Lipo}} + \Delta G^{\text{Packing}} + \Delta G^{\text{SolvGB}} + \Delta G^{\text{vdW}}$

where ΔG^{bind} is the total Prime energy, "Hbond" refers to the energy associated with hydrogen bonding, "Lipo" signifies lipophilic energy, "Packing" accounts for pi-pi packing correction, "SolvGB" represents the generalized Born electrostatic solvation energy, and "vdW" corresponds to the Van der Waals energy. These terms and their meanings are in accordance with previous works (Fatoki, 2023b; Zhang et al., 2017).

9.2.8 Arginase inhibition studies

Selected peptides (CQR, DR, DQR, QER, and REGNH) were synthesized ($\geq 95\%$ purity) by Genscript Corp (Piscataway, NJ, USA) and subjected to an *in vitro* arginase inhibition assay according to the method described by Gao et al. (2021). The synthetic peptide REGNH, a previously validated arginase inhibitor (Gao et al., 2021), was included as a positive control to verify assay performance and to provide a benchmark for evaluating the inhibitory activities of the lupin-derived peptides. Enzymatic activity was quantified by measuring urea formation. Reactions

were set up in 96-well microplates using a standard assay buffer composed of 100 mM sodium phosphate (pH 7.4) and 130 mM NaCl. Each well contained a total volume of 250 μ L, with arginase I from bovine liver (Millipore Sigma, Burlington, MA, USA) adjusted to a final concentration of 7 units/mL in assay buffer. Wells without enzyme served as negative controls. Peptide inhibitors (50 μ L) were added according to the experimental design at concentrations ranging from 10 μ g/mL to 100 μ g/mL. Reaction mixtures were pre-equilibrated for 30 min at 37 °C with gentle shaking (100 rpm), after which substrate solution (125 μ L) consisting of assay buffer with 100 mM L-arginine, 500 mM glycine, and 1.25 mM MnCl_2 was added. Incubations were performed for 1 h at the same temperature and shaking conditions and terminated by adding a stop reagent. The stop solution was prepared freshly by combining 2 parts of antipyrine in 50% (v/v) sulfuric acid (5 g/L) with 1 part of an oxime solution (0.8 g of 2,3-butanedione monoxime in 5% acetic acid). Following quenching, plates were kept protected from light at room temperature for at least 18 h to allow full color development, then heated to 45 °C for 4 h to enhance chromophore formation. The resulting brown coloration, proportional to urea concentration, was recorded by measuring absorbance at 466 nm using a Synergy H1 Hybrid Reader (BioTek Instruments, Winooski, VT, USA). Arginase I was selected for in vitro validation because recombinant human arginase II was not commercially available at the time of this study. To characterize enzyme kinetics, reactions were additionally performed across a range of L-arginine concentrations (12.5–800 mM). The initial velocities were fitted to the Michaelis–Menten model, and a double-reciprocal plot was used to determine K_m , V_{max} , and inhibition constant (K_i). The graphs were generated and fitted using GraphPad Prism version 9 (San Diego, CA, USA).

9.2.9 Statistical analysis

Statistical analyses were conducted using IBM SPSS statistical software version 28 (Chicago, IL, USA). All measurements were carried out in triplicate and are reported as mean $\pm$ standard deviation. Data were subjected to a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test to identify statistically significant differences ($p < 0.05$) among the mean values within each group.

9.3 RESULTS

The DrugBank database showed three compounds as potential inhibitors for arginase II (UniProt ID: P78540), which are DB00125 (arginine), DB03731 (S-2-(Boronoethyl)-L-Cysteine), and DB00129 (ornithine); while ten compounds were obtained for arginase I (UniProt ID: P05089) which are DB00129 (ornithine), DB03144 (N(5)-[(hydroxyamino)(imino)methyl]-L-ornithine), DB01983 (2(S)-amino-6-boronoheptanoic acid), DB02381 (nor-NOHA), DB02499 (Dinor-N(Omega)-Hydroxy-L-Arginine), DB04197 (Descarboxy-nor-N(Omega)-Hydroxy-L-Arginine), DB03731 (S-2-(Boronoethyl)-L-Cysteine), DB04530 (S,S-(2-Hydroxyethyl)Thiocysteine), DB02689 (S-{2-[Amino(Dihydroxy)-Lambda~4~-Sulfanyl]Ethyl}-D-Cysteine), and DB03904 (Urea). Chemical structure of compounds (DB series) and a synthetic peptide (REGNH) targeting arginase I and II are shown in Figure 9.1. The visual inspection of the structures of these compounds revealed the importance of arginine components in the scaffold end terminal as well as thiol group and carboxylic group at another end terminal.

The sequences of derivatives of the synthetic peptide (REGNH) were obtained by substituting amino acids E, G, N, and H based on the amino acid classes they belong to, except for proline, which was used to replace H. The 29 derivative sequences are listed in Table S1. The secondary derivative sequences were used to screen for potential white lupin proteins (are listed in Table S2), which were then subjected to *in silico* enzymatic hydrolysis. The result of chymotrypsin-trypsin-

pepsin simultaneous hydrolysis generated a lot of peptides, as shown in Table S3, which were then screened with pancreatic elastase (Table S2). The results of which after considering repeat peptides, 24 hydrolysate peptides that contained arginine at the C-terminal (Table 9.1) were selected and they indicate the presence of glutamic acid (E), aspartic acid (D), glutamine (Q), cysteine (C), and proline (P) at N-terminal.

The result of the ADME and allergenicity as shown in Table 9.1, indicate that all the 24 selected C-terminal arginine-containing lupin hydrolysate peptides possess the following features: molecular weight (MW) in the range of 271 to 789 g/mol, lipophilicity (Log P) range of -1.28 to -4.21, high solubility in water (Log S), low gastrointestinal absorption (GIA), no permeation across blood-brain barrier (BBB), no inhibition of cytochrome P450s (CYP450s), high skin permeation (log K_p) and no evidence of allergenicity. Moreover, the ADME results also showed that CR, QPR, PER, PPR, QEPR, PQCR, PPPR, and CPPPR peptides are not substrates of p-glycoprotein. The ADME profiles of the standard compound and synthetic peptide are also similar, except that some exhibit high GIA values.

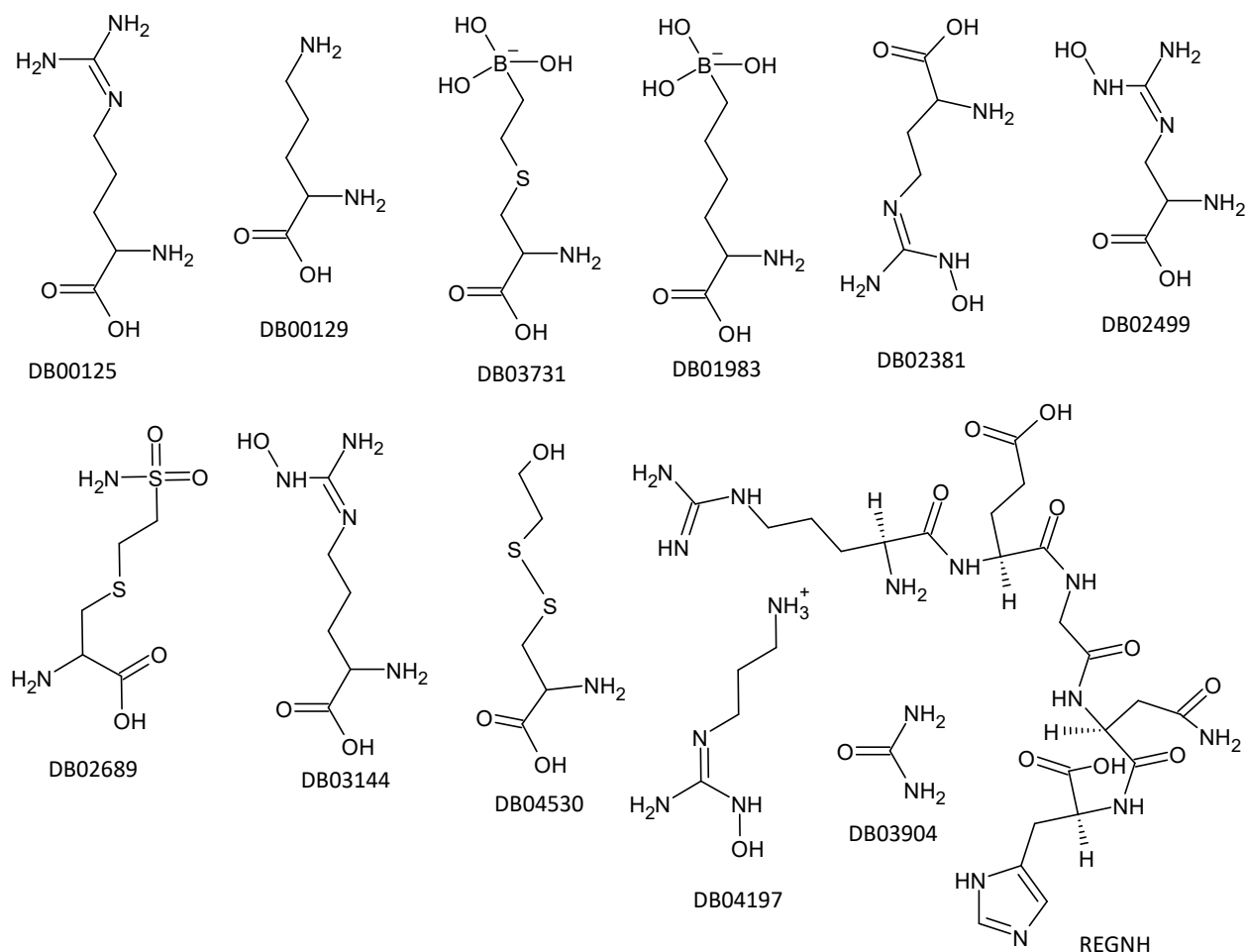


Figure 9.1: Chemical structure of chemical compounds (DB series) and synthetic peptide (REGNH) targeting arginase I and II.

The result of target prediction of all the 24 selected C-terminal arginine-containing hydrolysate peptides for possible activity showed that all the peptides containing proline (PR, QPR, PPR, QEPR, PQCR, PPPR, CPPPR, and PPPPPER) have no arginase I and II inhibitory activities (Table 9.2). Based on the p-value of the prediction, the order of arginase inhibitory activity was DR > CR > ER > QR > DDR > DER = CDR > QQQR > QQR and others. Moreover, the result showed that DB04197 and the synthetic peptide (REGNH) have no arginase inhibitory activity.

The result of molecular docking of all the 24 selected C-terminal arginine-containing hydrolysate peptides with respect to binding to arginase I and II enzymes showed that the order of summative

binding affinity was DR > QER > DQR > CQR > EER > PER > DER > DDR > others (Table 9.2). Moreover, the results showed that all peptides containing proline (P) could not bind to the arginases, while DB03144 and the synthetic peptide (REGNH) had binding affinities comparable to that of the hydrolysate peptide. The binding interaction of CQR, DQR, DR, and QER with arginase I and II are shown in Figures 9.2 and 9.3 while those of REGNH and compound DB03144 are in Figure 9.4. These interactions indicate the involvement of some active site amino acid residues such as His101, His126, Asn130, Ser137, Asp202, Asp 232, Asp253, Glu296, and others. Molecular dynamics simulations (MDS) were employed to forecast the structural stability of the protein and the binding status of the ligands in a physiological environment. The outcomes of the MDS study are shown in Figures 9.5 to 9.10. The results of RMSD of arginase I and II are shown in Figure 9.5, which indicate that binding interaction of CQR and DR with arginase II is more stable than that of Arginase I, while DQR is more stable with only arginase I over the period of simulation. Also in Figure 9.6, QER and REGNH were found to have stable interactions with both arginase I and II over the period of simulation (20-100 ns), while DB03144 was stable with only arginase I. Overall, DQR and DB03144 were found to be unstable with arginase II due to the ligand RMSDs of approximately 64 Å and 54 Å, respectively.

The results of the RMSF of arginase I and II are shown in Figures 9.7 and 9.8. For arginase I, similarity exists in the interaction with DR and QER, DQR and REGNH, as well as for CQR and DB03144, while for arginase II, similarity exists in the interaction with CQR, DR, DQR, QER, and DB03144. The results of protein-ligand interactions (or contacts) are presented in Figures 9.9 and 9.10, which indicate various amino acid residues that formed hydrogen bonds, hydrophobic, ionic, and water bridges. Specifically, CQR, DR, DQR, QER, DB03144, and REGNH interacted through ionic bridges with arginase II than arginase I. Overall, the results of protein-ligand

interactions validated the amino acid residues present in the docking interactions, as shown in Figures 9.2-9.4. Additionally, the results for both arginase I and II showed a $R_g \leq 0.6 \text{ \AA}$, and the SASA was approximately $1500 \text{ \AA}^2$. The Figures for R_g and SASA results are provided in Figures S1-S4.

The binding free energies of all complexes were computed using MMGBSA at both 0 ns and 100 ns time points as shown in Table 9.3. Overall, binding energy ΔG^{bind} (Total) at 100 ns was lower than that 0 ns, except for the interaction of arginase I with DR (-17.7 to -35.0 kcal.mol⁻¹), as well as arginase II with DR (-27.0 to -27.7 kcal.mol⁻¹), QER (-33.2 to -61.2 kcal.mol⁻¹) and REGNH (-55.7 and -86.7 kcal.mol⁻¹) that showed increase in binding energy. Also, arginase I showed relative stable interaction with DQR (-56.8 to -51.8 kcal.mol⁻¹) and QER (-46.6 to -45.3 kcal.mol⁻¹).

The *in vitro* arginase inhibitory activities of the synthesized peptides (CQR, DR, DQR, QER, and REGNH) are shown in Figure 9.11A. All peptides exhibited concentration-dependent inhibition, with DQR and QER showing the highest inhibitory activity (approximately 16–18% inhibition at 100 $\mu\text{g/mL}$), whereas CQR showed no arginase inhibitory activity at any concentration. The Lineweaver-Burk double-reciprocal plots for DQR, QER, and REGNH (Figure 11B–D) revealed mixed-type inhibition, with lines converging near the y-axis at different x-intercepts, indicating increased apparent K_m and decreased V_{max} . The kinetic analysis yielded K_i values of 150 μM for DQR, 330 μM for QER, and 320 μM for REGNH, establishing the inhibitory potency ranking as $\text{DQR} > \text{REGNH} > \text{QER}$.

Table 9.1: ADME properties of standard arginase inhibitory compounds and the hydrolysate peptides obtained from *in silico* digestion of white lupin proteins with chymotrypsin, trypsin, pepsin and elastase

S.N	HYDROLYSATE PEPTIDES	Predicted ADME Parameter													
		MW	MR	TPSA (Å ²)	Log P	ESOL Log S	ESOL Class	GIA	BBB	P-gp	CYPs Inhibitor	Log Kp (cm/s)	BS	SA	Allergenicity
1	CR	277.34	69.89	193.12	-1.78	1.71	Highly soluble	Low	No	No	No	-10.94	0.55	3.03	NE
2	ER	303.31	73.34	191.62	-2.04	1.96	Highly soluble	Low	No	Yes	No	-11.41	0.55	3.15	NE
3	QR	302.33	74.48	197.41	-2.35	2.38	Highly soluble	Low	No	Yes	No	-11.86	0.55	3.15	NE
4	DR	289.29	68.54	191.62	-2.48	2.2	Highly soluble	Low	No	Yes	No	-11.57	0.55	3.13	NE
5	PR	271.32	73.47	140.33	-1.59	1.94	Highly soluble	Low	No	Yes	No	-11.19	0.55	3.03	NE
6	EQR	431.44	103.29	263.81	-2.92	2.45	Highly soluble	Low	No	Yes	No	-13.19	0.17	4.05	NE
7	DDR	404.38	92.54	258.02	-2.95	2.53	Highly soluble	Low	No	Yes	No	-13.07	0.17	3.94	NE
8	DER	418.40	97.34	258.02	-2.79	2.29	Highly soluble	Low	No	Yes	No	-12.91	0.17	4.00	NE
9	CDR	392.43	93.89	259.52	-2.72	2.04	Highly soluble	Low	No	Yes	No	-12.44	0.17	3.95	NE
10	CQR	405.47	99.83	265.31	-2.5	2.21	Highly soluble	Low	No	Yes	No	-12.73	0.17	3.93	NE
11	QPR	399.45	103.41	217.72	-2.69	2.51	Highly soluble	Low	No	No	No	-13.13	0.17	3.93	NE

12	DQR	417.42	98.48	263.81	- 3.28	2.7	Highly soluble	Low	No	Yes	No	-13.36	0.17	4.02	NE
13	PER	400.43	102.28	206.73	- 2.21	2.21	Highly soluble	Low	No	No	No	-12.73	0.17	3.96	NE
14	EER	432.43	102.15	258.02	- 2.59	2.04	Highly soluble	Low	No	Yes	No	-12.74	0.17	4.03	NE
15	QER	431.44	103.29	263.81	- 2.83	2.45	Highly soluble	Low	No	Yes	No	-13.19	0.17	4.07	NE
16	PPR	368.43	102.4	160.64	- 1.28	0.74	Highly soluble	Low	No	No	No	-10.95	0.55	3.78	NE
17	QQR	430.46	104.42	269.6	- 3.29	2.87	Highly soluble	Low	No	Yes	No	-13.65	0.17	4.05	NE
18	QEPR	528.56	132.22	284.12	- 3.46	2.9	Highly soluble	Low	No	No	No	-14.82	0.17	4.77	NE
19	PQCR	502.59	128.76	280.42	- 2.87	2.76	Highly soluble	Low	No	No	No	-14.4	0.17	4.72	NE
20	QQQR	558.59	134.37	341.79	- 4.21	3.36	Highly soluble	Low	No	Yes	No	-15.44	0.17	4.87	NE
21	DQER	546.53	127.29	330.21	- 3.83	2.78	Highly soluble	Low	No	Yes	No	-14.69	0.17	4.81	NE
22	PPPR	465.55	131.33	180.95	- 1.23	0.24	Highly soluble	Low	No	No	No	-11.51	0.17	4.46	NE
23	CPPPR	568.69	156.68	254.05	- 1.83	0.21	Highly soluble	Low	No	No	No	-12.61	0.17	5.14	NE
24	PPPPPER	788.89	218	287.97	- 2.12	-0.69	Very soluble	Low	No	Yes	No	-13.95	0.17	6.72	NE
SN	STANDARD COMPOUNDS	MW	MR	TPSA (Å ²)	Log P	ESOL Log S	ESOL Class	GIA	BBB	P- gp	CYPs Inhibitor	Log Kp	BS	SA	Allergenicity

(cm/s)															
1	DB00125	174.2	44.54	127.72	- 2.08	2.05	Highly soluble	Low	No	No	No	-10.34	0.55	2.47	ND
2	DB00129	132.16	33.34	89.34	- 1.79	2.38	Highly soluble	High	No	No	No	-10.24	0.55	1.61	ND
3	DB03731	210.04	49.57	149.31	- 2.72	1.7	Highly soluble	Low	No	No	No	-10.34	0.56	2.92	ND
4	DB01983	192.00	46.79	124.01	- 2.45	1.55	Highly soluble	High	No	No	No	-9.93	0.56	2.34	ND
5	DB02381	176.17	40.55	133.96	- 2.59	2.32	Highly soluble	Low	No	No	No	-10.67	0.55	2.67	ND
6	DB02499	162.15	35.74	133.96	- 2.87	2.57	Highly soluble	Low	No	No	No	-10.84	0.55	2.96	ND
7	DB02689	228.29	49.89	157.16	- 2.14	1.67	Highly soluble	Low	No	No	No	-10.54	0.55	3.38	ND
8	DB03144	190.2	45.36	133.96	- 2.39	2.08	Highly soluble	Low	No	No	No	-10.51	0.55	2.66	ND
9	DB03904	60.06	12.92	69.11	- 0.99	0.69	Highly soluble	High	No	No	No	-7.68	0.55	1.00	ND
10	DB04197	133.17	35.23	98.28	- 1.91	0.85	Highly soluble	High	No	No	No	-8.53	0.55	2.49	ND
11	DB04530	197.28	46.97	134.15	- 0.98	0.7	Highly soluble	High	No	No	No	-9.04	0.55	3.09	ND
12	REGNH	611.61	145.15	350.69	- 4.02	2.66	Highly soluble	Low	No	Yes	No	-15.36	0.17	5.31	NE

Legend: **Drug-likeness:** Bioavailability Score (BS). **Lipophilicity:** Consensus Log P. **Medicinal chemistry:** Synthetic accessibility (SA). **Pharmacokinetics:** Gastrointestinal absorption (GIA), Blood-brain barrier (BBB), P-glycoprotein (P-gp) substrate, Inhibition of Cytochrome P450 (CYPs) type CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4, Skin permeation (Log Kp). **Physicochemical**

properties: Molecular weight (MW), Molar Refractivity (MR), Total polar surface area (TPSA). **Water solubility:** ESOL Log S, ESOL Class. **NE:** no evidence. **ND:** not determined.

Table 9.2: Predicted arginase I and II inhibitory activities and binding energy of interactions of standard compounds and the hydrolysate peptides obtained from *in silico* digestion of white lupin protein with chymotrypsin, trypsin, pepsin, and elastase

SN	HYDROLYSATE PEPTIDES	Target Genes (P-value)	ARGINASE I (PDB ID: 2AEB) Binding Affinity (kcal.mol ⁻¹)	ARGINASE II (PDB ID: 4IXU) Binding Affinity (kcal.mol ⁻¹)
1	CR	ARG2 (5.17e-42), ARG1 (5.24e-27)	-6.6	-5.1
2	ER	ARG2 (4.05e-41), ARG1 (1.94e-26)	-5.4	-5.653
3	QR	ARG2 (4.05e-41), ARG1 (1.94e-26)	-7.0	-7.7
4	DR	ARG2 (6.00e-43), ARG1 (1.33e-27)	-7.2	-8.2
5	PR	NO ARGINASE ACTIVITY	-5.1	-6.6
6	EQR	ARG2 (1.73e-36), ARG1 (1.73e-23)	-5.3	-7.5
7	DDR	ARG2 (2.90e-40), ARG1 (6.79e-26)	-6.8	-6.9
8	DER	ARG2 (6.69e-38), ARG1 (2.15e-24)	-6.8	-7.1
9	CDR	ARG2 (6.69e-38), ARG1 (2.15e-24)	-6.0	-7.3
10	CQR	ARG2 (6.57e-36), ARG1 (1.73e-23)	-7.1	-7.4
11	QPR	NO ARGINASE ACTIVITY	-6.3	-6.6
12	DQR	ARG2 (3.56e-37), ARG1 (6.24e-24)	-8.1	-6.7
13	PER	NO ARGINASE ACTIVITY	-6.6	-7.3
14	EER	ARG2 (1.18e-38), ARG1 (7.14e-25)	-7.0	-7.3
15	QER	ARG2 (1.78e-36), ARG1 (1.73e-23)	-7.2	-7.6
16	PPR	NO ARGINASE ACTIVITY	-7.7	-6.4
17	QQR	ARG2 (1.18e-38), ARG1 (7.14e-25)	-6.1	-7.6
18	QEPR	NO ARGINASE ACTIVITY	-6.5	-6.2

19	PQCR	NO ARGINASE ACTIVITY	-5.5	-6.0
20	QQQR	ARG2 (1.18e-38), ARG1 (7.14e-25)	-6.4	-5.6
21	DQER	ARG2 (8.33e-36), ARG1 (4.63e-23)	-6.7	-5.1
22	PPPR	NO ARGINASE ACTIVITY	-6.5	-6.6
23	CPPPR	NO ARGINASE ACTIVITY	-7.4	-6.1
24	PPPPPER	NO ARGINASE ACTIVITY	-7.425	-8.179
SN	STANDARD COMPOUND	Target Genes (P-value)	ARGINASE I (PDB ID: 2AEB) Binding Affinity (kcal.mol ⁻¹)	ARGINASE II (PDB ID: 4IXU) Binding Affinity (kcal.mol ⁻¹)
1	DB00125	ARG2 (1.93e-45), ARG1 (3.39e-57)	-5.3	-6.6
2	DB00129	ARG2 (1.26e-52), ARG1 (1.51e-65)	-4.7	-4.9
3	DB03731	ARG2 (1.33e-36), ARG1 (1.44e-23)	ND	ND
4	DB01983	ARG2 (1.03e-46), ARG1 (1.09e-58)	ND	ND
5	DB02381	ARG2 (2.74e-38), ARG1 (6.99e-59)	-6.0	-4.6
6	DB02499	ARG2 (3.07e-50), ARG1 (3.09e-32)	-6.0	-4.5
7	DB02689	ARG2 (7.55e-40), ARG1 (1.25e-25)	-4.7	-5.7
8	DB03144	ARG2 (3.07e-54), ARG1 (2.35e-60)	-5.6	-7.2
9	DB03904	NOT DETERMINABLE	-3.7	-4.1
10	DB04197	NO ARGINASE ACTIVITY	-5.5	-6.0
11	DB04530	ARG2 (1.38e-38), ARG1 (7.91e-25)	-4.8	-5.6
12	REGNH	NO ARGINASE ACTIVITY	-6.8	-7.7

*Standard deviation of ± 0.2 . ND: Not Determined, this was because Atom type B in the chemical structure was not a valid in AutoDock.

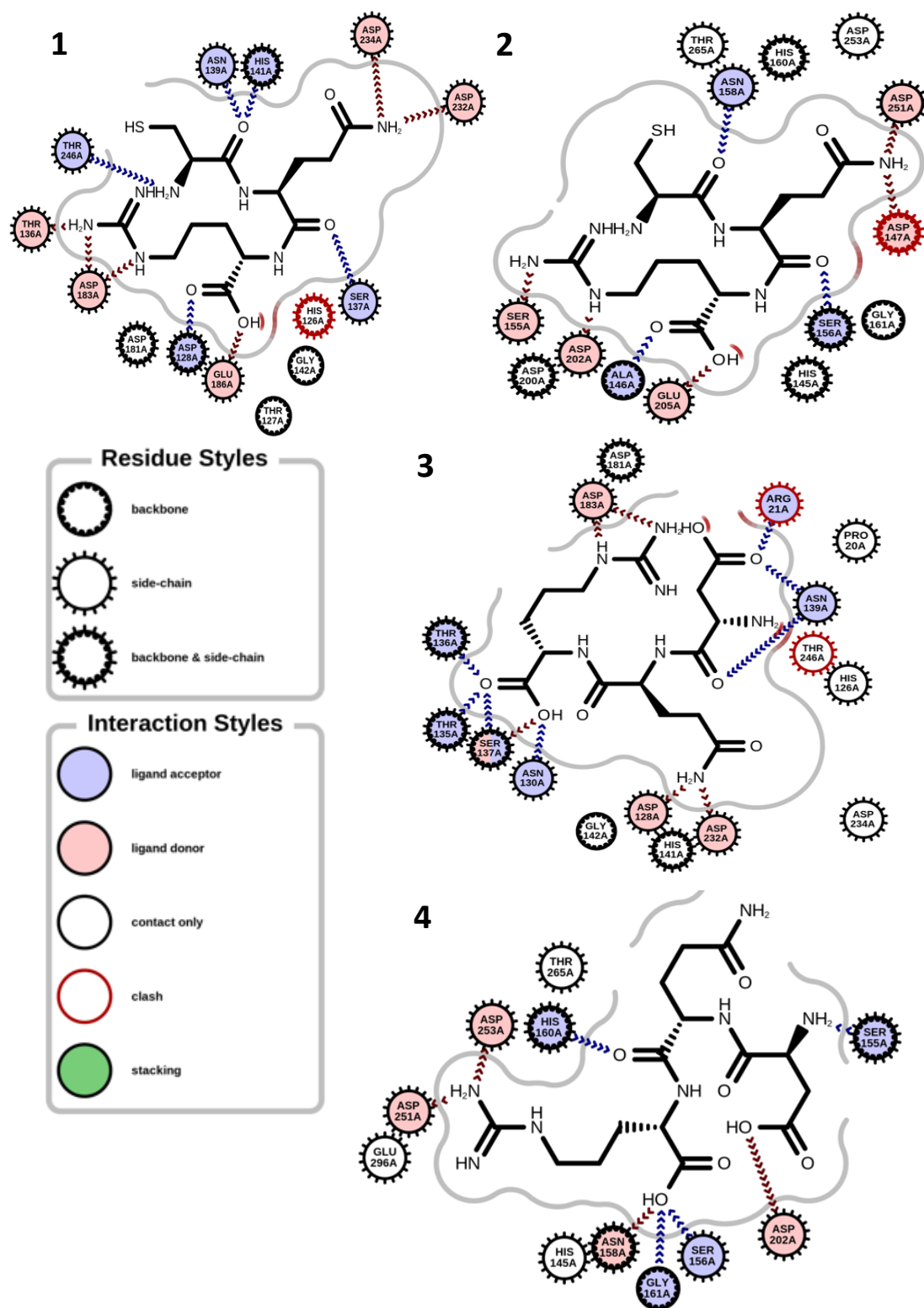


Figure 9.2 Docking interaction of (1) CQR with arginase I (PDB ID: 2AEB). (2) CQR with arginase II (PDB ID: 4IXU). (3) DQR with arginase I (PDB ID: 2AEB). (4) DQR with arginase II (PDB ID: 4IXU).

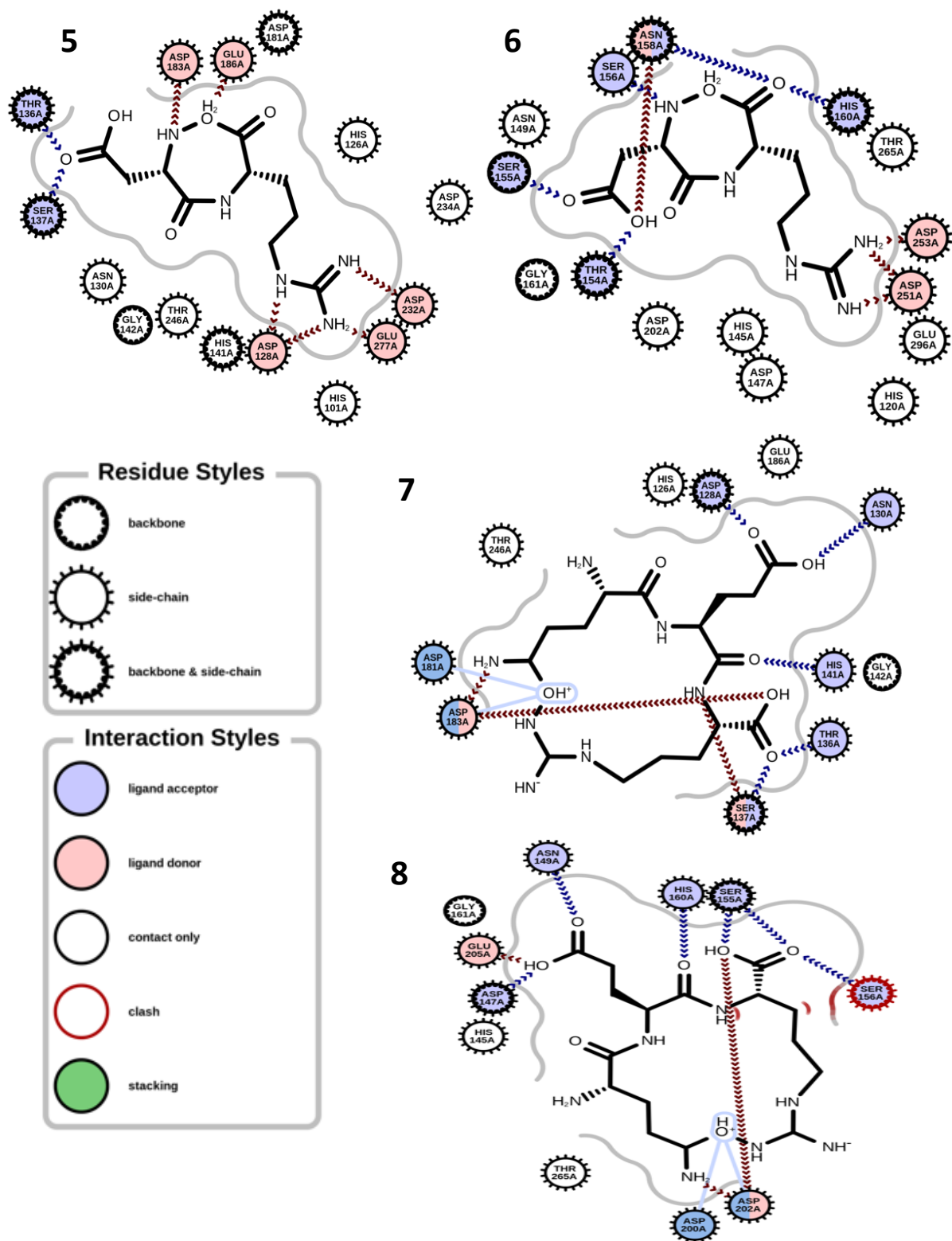


Figure 9.3 Docking interaction of (5) DR with arginase I (PDB ID: 2AEB). (6) DR with arginase II (PDB ID: 4IXU). (7) QER with arginase I (PDB ID: 2AEB). (8) QER with arginase II (PDB ID: 4IXU).

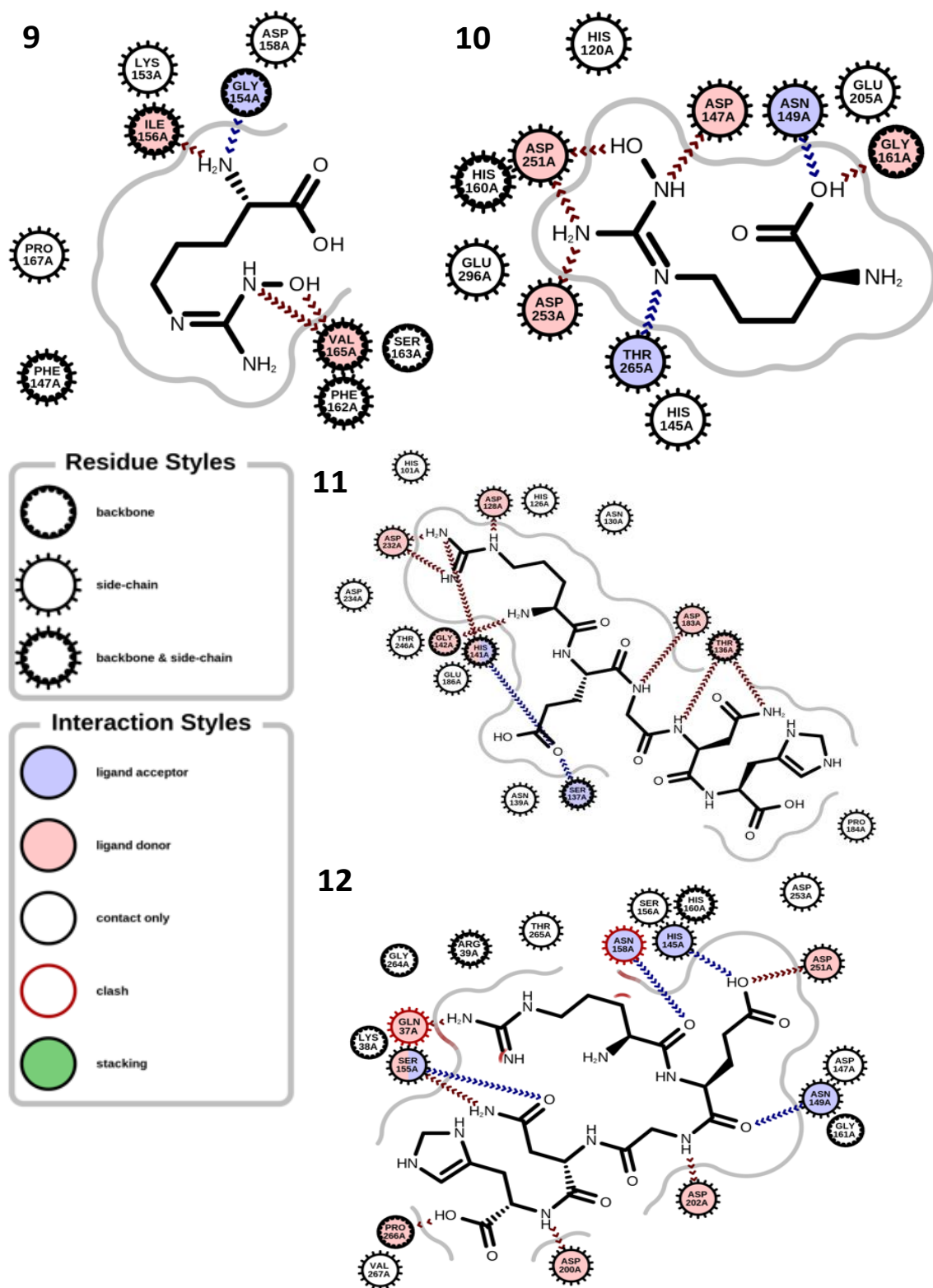


Figure 9.4 Docking interaction of (9) DB03144 with arginase I (PDB ID: 2AEB). (10) DB03144 with arginase II (PDB ID: 4IXU). (11) REGNH with arginase I (PDB ID: 2AEB). (12) REGNH with arginase II (PDB ID: 4IXU).

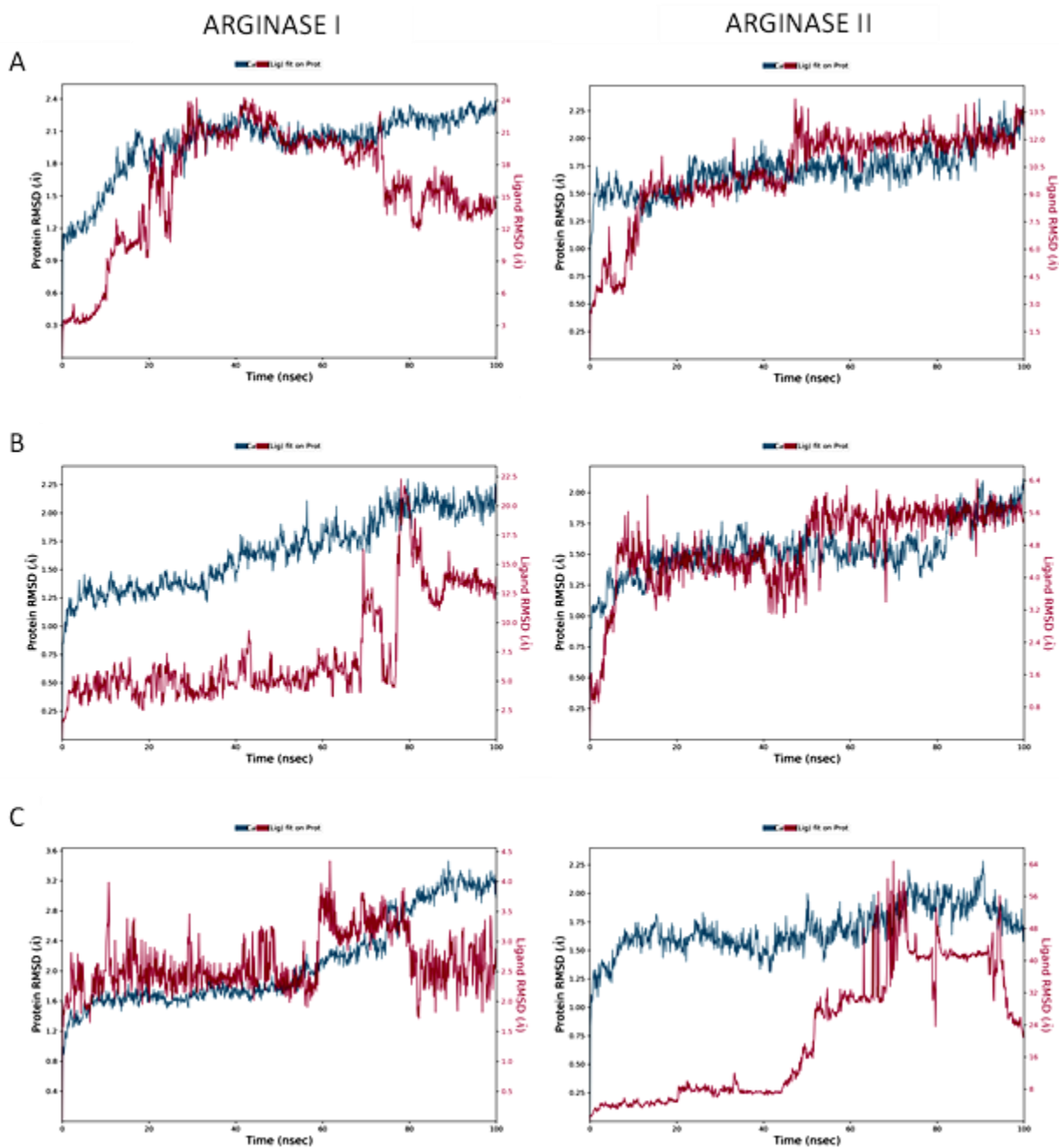


Figure 9.5 RMSD of arginase I and arginase II on binding with (A) CQR. (B) DR (C) DQR. Color key: blue-Protein; red-Ligand.

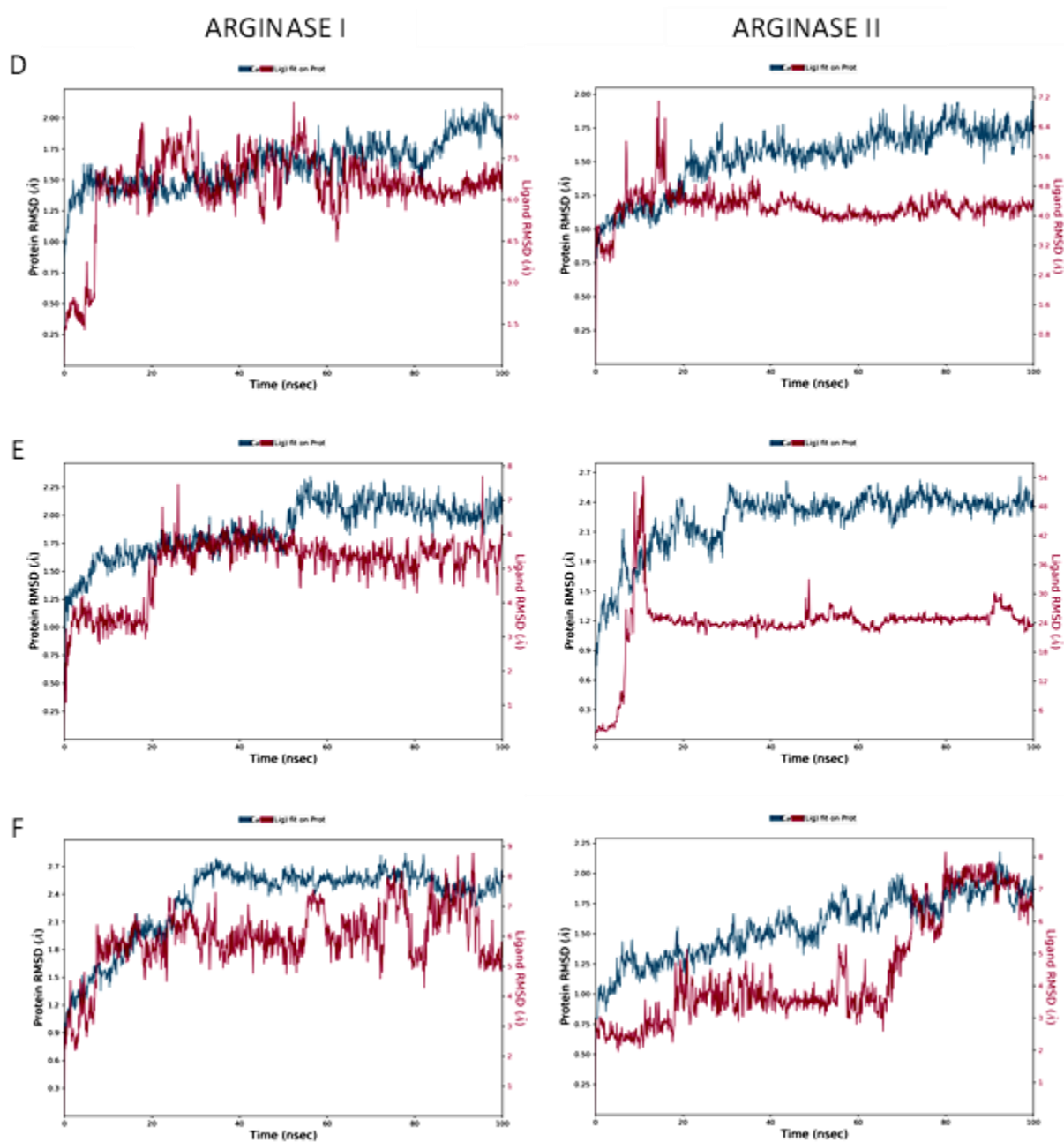


Figure 9.6 RMSD of arginase I and arginase II on binding with (D) QER. (E) DB03144. (F) REGNH. Color key: blue-Protein; red-Ligand.

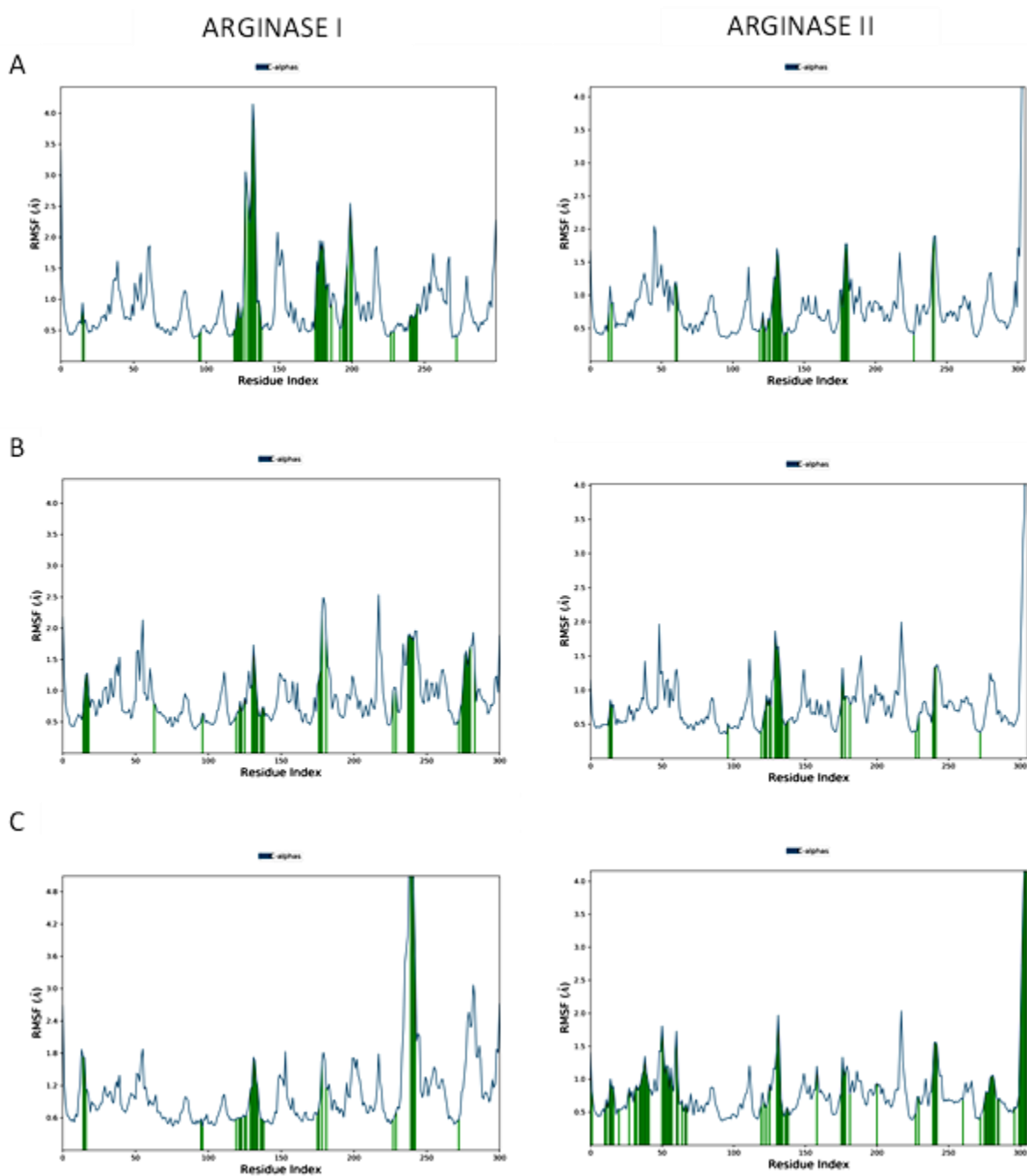


Figure 9.7 RMSF of arginase I and arginase II on binding with (A) CQR (B) DR (C) DQR

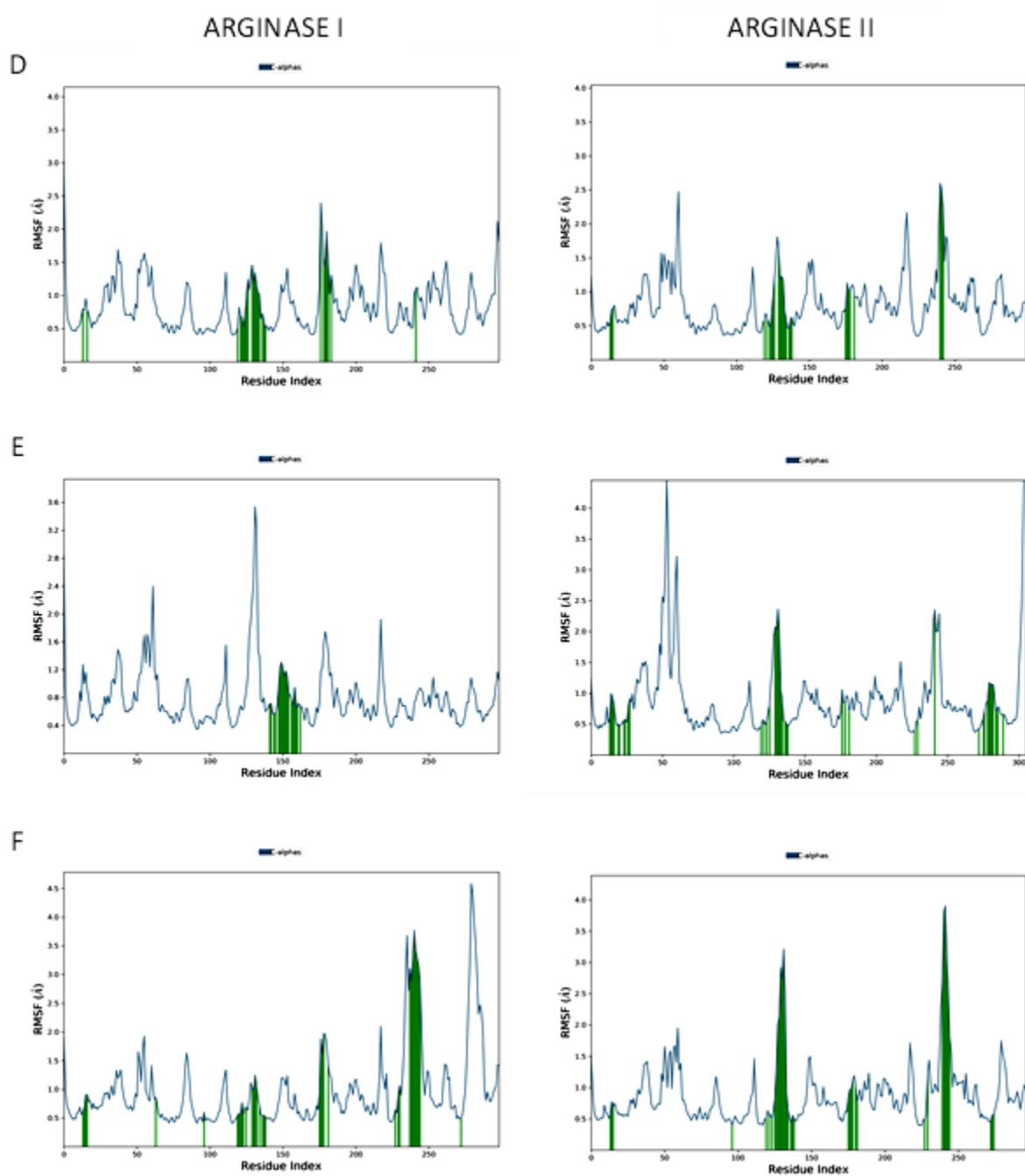


Figure 9.8 RMSF of arginase I and arginase II on binding with (D) QER (E) DB03144 (F) REGNH

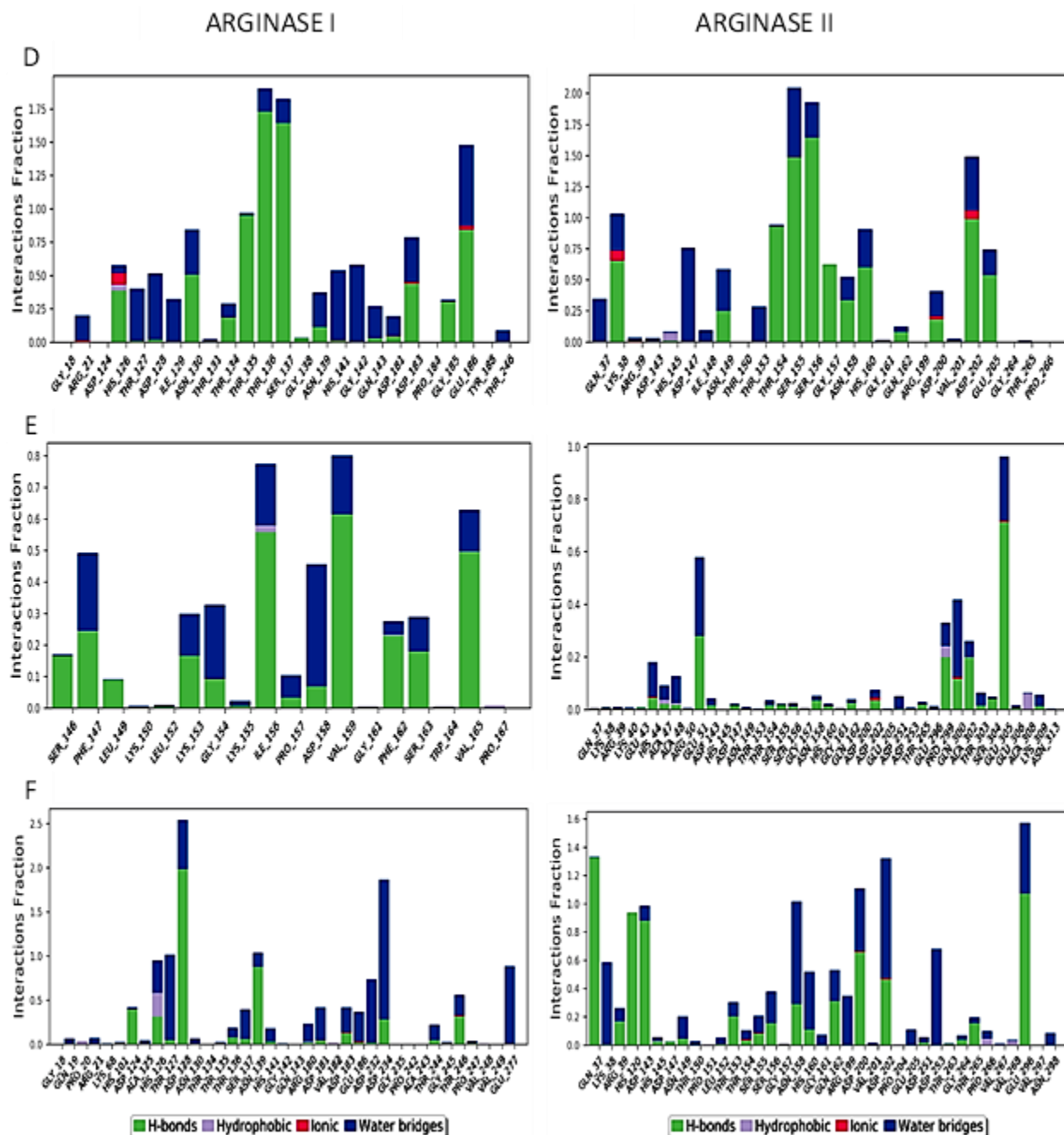


Figure 9.10 Interaction profile of the contact of arginase I and arginase II on binding with (D) QER (E) DB03144 (F) REGNH

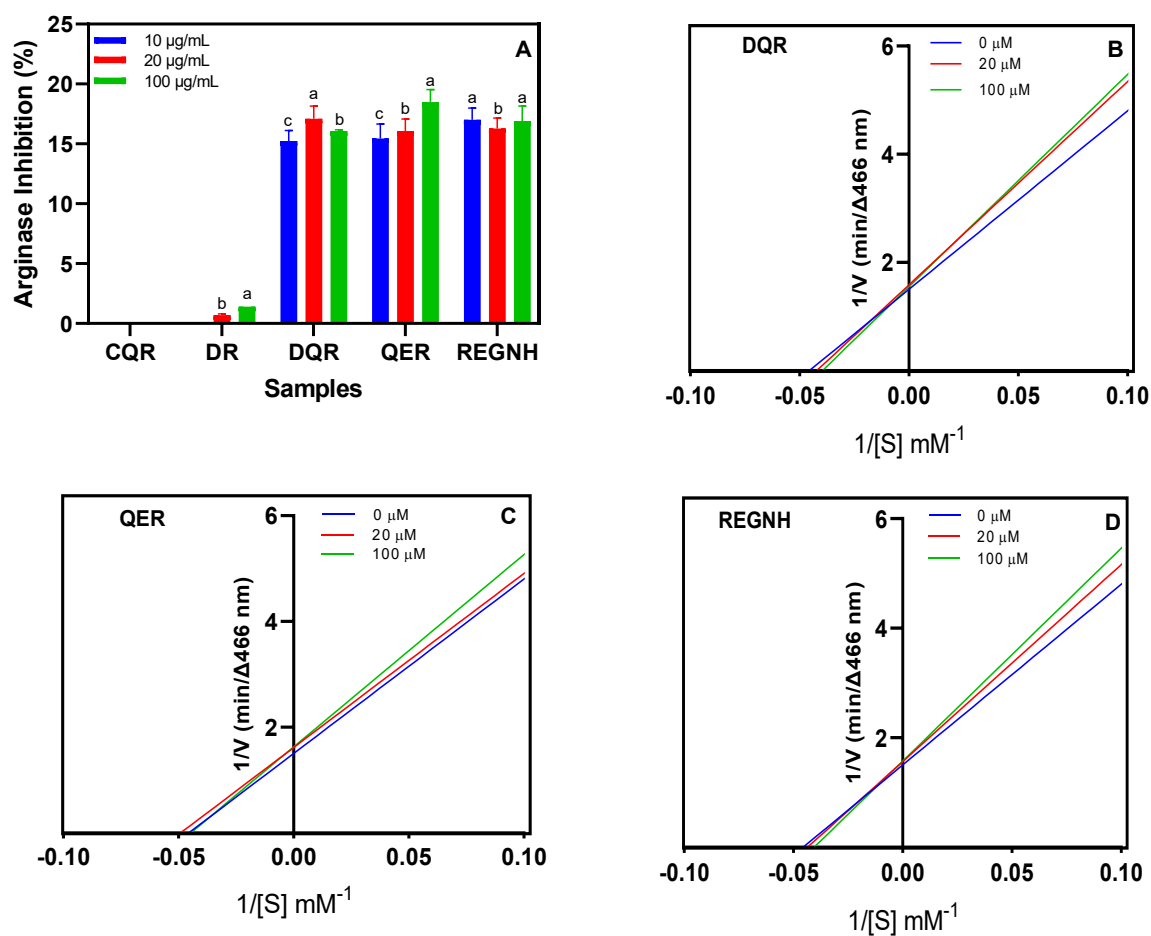


Figure 9.11 (A) In vitro arginase inhibitory properties of CQR, DR, DQR, QER, and REGNH peptides. Lineweaver-Burk plots showing kinetics of arginase inhibition by (B) DQR, (C) QER, and (D) REGNH peptides.

Table 9.3 Prime molecular mechanics/generalized Born surface area (MMGBSA) binding energy of interaction of Arginase I and Arginase II with the selected peptides and standard compounds, before and after molecular dynamics simulation.

Protein	Ligand	Simulation Time (ns)	MMGBSA ΔG (kcal.mol ⁻¹)							
			Coulomb	Covalent	Hbond	Lipo	Packing	Solv_GB	vdW	ΔG^{bind} (Total)
Arginase I	CQR	0	-59.0	7.2	-12.2	-14.8	0	66.9	-40.9	-52.8
		100	-78.5	2.7	-10.1	-6.9	0	64.0	-11.1	-39.9
	DR	0	-38.0	7.7	-4.9	-6.6	0	50.4	-26.2	-17.7
		100	-39.3	5.4	-2.4	-7.9	0	25.8	-16.6	-35.0
	DQR	0	-39.9	2.6	-9.9	-11.2	0	46.3	-44.7	-56.8
		100	-46.1	7.3	-7.2	-12.5	0	45.6	-38.9	-51.8
	QER	0	-90.9	5.1	-11.9	-11.6	0	98.4	-35.7	-46.6
		100	-68.2	9.7	-5.8	-15.6	0	68.8	-34.2	-45.3
	DB03144	0	-15.9	-4.1	-1.9	-17.4	0	15.3	-25.7	-49.7
		100	-14.3	-0.6	-0.8	-6.9	0	12.9	-17.5	-27.3
Arginase II	CQR	0	-71.8	9.0	-15.1	-14.9	0	70.1	-39.4	-62.0
		100	-17.5	3.1	-5.0	-12.3	0	18.1	-21.7	-35.2
	DR	0	-46.1	9.6	-3.9	-9.0	0	50.6	-28.1	-27.0
		100	-43.0	10.4	-4.8	-9.9	0	46.6	-27.0	-27.7
	DQR	0	-58.8	5.3	-8.9	-14.0	0	47.4	-36.3	-65.3
		100	-31.4	2.7	-2.1	-9.3	0	18.1	-25.8	-47.9
	QER	0	-51.8	3.9	-12.0	-9.3	0	66.7	-30.7	-33.2
		100	-79.7	2.8	-13.2	-12.5	0	71.0	-29.5	-61.1
	DB03144	0	-18.0	-3.3	-3.7	-11.6	0	23.2	-31.2	-44.6
		100	-6.3	-0.3	-0.5	-11.3	0	15.6	-21.0	-23.7
Arginase II	REGNH	0	-68.9	9.3	-6.5	-11.2	0	63.7	-42.2	-55.7
		100	-73.0	6.4	-8.3	-18.7	0	59.5	-52.7	-86.7

Legend: **Total**: Total energy (Prime energy). **Covalent**: Covalent binding energy. **Coulomb**: Coulomb energy. **Hbond**: Hydrogen bonding energy. **Lipo**: Lipophilic energy. **Solv GB**: Generalized Born electrostatic solvation energy. **Packing**: Pi-pi packing correction. **vdW**: Van der Waals energy.

Table 9.4 Kinetic parameters for the inhibition of arginase activity by DQR, QER, and REGNH peptides

DQR	Control	20 μM	100 μM
K_m or K'_m (mM)	31.2	35.3	35.4
V_{max} or V'_{max}	0.72	0.70	0.70
K_i (μM)		~150 μ M	
QER	Control	20 μM	100 μM
K_m or K'_m (mM)	31.2	33.1	36.2
V_{max} or V'_{max}	0.72	0.69	0.69
K_i (μM)		~330 μ M	
REGNH	Control	20 μM	100 μM
K_m or K'_m (mM)	31.2	33.1	37.1
V_{max} or V'_{max}	0.72	0.70	0.71
K_i (μM)		~320 μ M	

9.4 DISCUSSION

9.4.1 Peptide assembly

The computational screening strategy based on REGNH derivatives and C-terminal arginine motifs identified peptides with potential arginase-modulatory activity. Structural inspection of known arginase inhibitors from DrugBank revealed the importance of arginine-like structures with terminal carboxylate or thiol groups (Cama et al., 2003; Mortier et al., 2017). This guided the design of 29 REGNH derivative sequences, which were used to screen the white lupin genome and were subsequently subjected to simulated gastrointestinal digestion with chymotrypsin, trypsin, pepsin, and elastase. The *in-silico* hydrolysis yielded 24 unique C-terminal arginine peptides characterized by enrichment of acidic residues (aspartate and glutamate), polar uncharged residues (glutamine), and cysteine at N-terminal or internal positions. Notably absent were hydrophobic or aromatic residues, suggesting that the lupin proteome's amino acid distribution and the chosen digestion enzymes favor the generation of charged, hydrophilic peptides. The presence of C-terminal arginine is functionally significant, as previous studies have demonstrated that peptides with this motif exhibit diverse bioactivities including inhibition of cholinesterases (Asen et al., 2023), furin (Fatoki et al., 2022), and ACE (Iroyukifujita et al., 2000; Zhao et al., 2020).

9.4.2 Pharmacokinetic profiling

ADME screening indicated that all 24 C-terminal arginine peptides are hydrophilic, highly water-soluble, and exhibit low predicted gastrointestinal absorption (Table 9.1). No peptides showed evidence of allergenicity or cytochrome P450 inhibition. All proline-containing peptides showed no predicted arginase inhibitory activity despite some exhibiting more favorable P-glycoprotein profiles, suggesting that proline's cyclic structure may prevent productive arginase binding. It should be noted that SwissADME was developed for small organic molecules, and its reliability for short charged peptides remains uncertain.

9.4.3 Target prediction

Similarity Ensemble Approach (SEA) target prediction, which identifies protein targets based on chemical similarity to known ligands (Keiser et al., 2007), was applied as an exploratory screening step to prioritize candidate peptides for downstream computational analysis. Of the 24 C-terminal arginine peptides, 15 were predicted to have arginase I and II inhibitory activity, with a predicted activity hierarchy of DR > CR > ER > QR > DDR > DER = CDR > QQQR > QQR and others (Table 9.2). All eight proline-containing peptides showed no predicted arginase inhibitory activity. However, the predictive reliability of SEA for short peptide ligands was limited. Notably, the synthetic peptide REGNH, a validated arginase inhibitor (Gao et al., 2021), was not predicted to have arginase inhibitory activity, indicating that binding modes or allosteric mechanisms not represented in the SEA training dataset are overlooked by this ligand-similarity approach. Furthermore, subsequent experimental validation revealed a poor correlation between SEA rankings and functional inhibition: DR, the top-ranked peptide, showed negligible activity (<2%), while QER, ranked 15th, achieved the highest inhibition (~18%). These discrepancies confirm that SEA predictions served as one of several complementary filters for candidate prioritization rather than as reliable predictors of functional enzyme inhibition.

9.4.4 Molecular docking

Molecular docking of the 16 predicted-active peptides against arginase I (PDB: 2AEB) and arginase II (PDB: 4IXU) yielded binding affinities of ≤ -5.0 kcal/mol for most peptides (Table 9.2). The overall binding affinity ranking was DR > QER > DQR > CQR > EER > PER > DER > DDR, among others. This ranking differed from the SEA target prediction hierarchy, with QER (ranked 15th in target prediction) achieving the second-best docking score. Interaction analysis identified the engagement of active-site residues involved in substrate recognition and catalysis, including His101, His126, Asn130, Asp181, Asp232, Asp253, and Glu296 (Mortier et al., 2017).

The reference compound DB03144 exhibited binding affinity comparable to that of the hydrolysate peptides, while the boronic acid-containing inhibitor DB03904 could not be evaluated due to limitations in parameterizing boron atoms within the force field. Importantly, AutoDock Vina's scoring function does not explicitly model coordination interactions with the binuclear manganese cluster, which is critical for arginase inhibition (Kanyo et al., 1996). The reported binding affinities therefore reflect general protein-ligand interactions rather than specific metal-coordination geometry, and this limitation likely contributes to the discrepancies observed between predicted binding affinities and experimental inhibitory activities. Indeed, subsequent experimental validation confirmed that docking rankings did not reliably predict functional inhibition: DR, the top-ranked peptide, showed negligible activity (<2%), while lower-ranked peptides (DQR, QER) achieved significant inhibition. These findings underscore that docking-derived binding affinities should be interpreted cautiously and used as a prioritization tool rather than as quantitative predictors of inhibitory potency.

9.4.5 Molecular dynamics simulations

Molecular dynamics simulations over 100 ns were performed for top-ranked peptides (CQR, DR, DQR, QER) and controls (DB03144, REGNH) in complex with both arginase isoforms to assess binding stability (Figures 9.5 and 9.6). RMSD analysis revealed isoform-specific stability patterns. CQR showed stable binding to both isoforms (RMSD < 3 Å), while DR exhibited contrasting behavior: moderate fluctuations with arginase I but consistent stability with arginase II (ligand RMSD < 0.5 Å), suggesting possible preferential binding to arginase II. DQR maintained moderate stability with arginase I (RMSD 1.5–2.5 Å) but showed ligand dissociation from arginase II after ~60 ns (RMSD ~10 Å). QER maintained stable interactions with both isoforms throughout the simulation (RMSD < 3 Å). DB03144 was stable with arginase I but unstable with arginase II,

whereas REGNH maintained interactions with both isoforms, consistent with its reported dual-isoform activity (Gao et al., 2021). These computationally inferred isoform preferences remain to be experimentally verified, as only arginase I was available for in vitro testing.

RMSF analysis (Figures 9.7 and 9.8) indicated that arginase I exhibited greater ligand-dependent flexibility, with different peptides inducing distinct fluctuation patterns in the substrate-binding pocket and active-site loop regions. In contrast, arginase II exhibited more uniform responses across ligands, suggesting a more structurally constrained active-site architecture (Failla et al., 2024). Protein-ligand contact analysis (Figures 9.9 and 9.10) showed that hydrogen bonding was the dominant interaction type across most complexes, consistent with the charged, hydrophilic nature of C-terminal arginine peptides. Ionic interactions were more prominent in arginase II complexes. The active-site residues most frequently engaged were His101, His126, Asn130, Asp181, Asp232, and Glu277, which are known to participate in substrate recognition and catalysis (Ash, 2004). Radius of gyration ($R_g \leq 0.6 \text{ \AA}$) and SASA ($\sim 1500 \text{ \AA}^2$) remained stable across all systems (Figures S1–S4). It should be noted that the MD simulations were performed as single 100 ns trajectories without replicates, and the OPLS-2005 force field used was not specifically parameterized for short charged peptides or for explicit modeling of binuclear manganese coordination. The RMSD/RMSF observations, therefore, represent computational indicators of relative binding stability rather than evidence of a biological mechanism.

9.4.6 Binding energy of interaction of Arginase I and Arginase II

MMGBSA binding free energies were calculated at 0 ns and 100 ns to evaluate relative changes in binding stability during simulation (Table 9.3). For arginase I, DR showed increased binding energy (-17.7 to -35.0 kcal/mol), while DQR (-56.8 to -51.8 kcal/mol) and QER (-46.6 to -45.3 kcal/mol) remained relatively stable. For arginase II, QER showed substantial stabilization (-33.2

to -61.2 kcal/mol), while DQR showed destabilization (-65.3 to -47.9 kcal/mol), correlating with the ligand dissociation observed in RMSD analysis. Electrostatic interactions were the major favorable contributors, partially offset by unfavorable polar solvation energies, consistent with highly charged peptide-protein systems. Notably, DR's favorable arginase I binding energy did not translate to experimental activity ($<2\%$ inhibition), illustrating the disconnect between computed binding energies and functional inhibition. MMGBSA values are semi-quantitative estimates that do not account for entropic contributions and are best interpreted as relative comparisons between systems rather than absolute measures of binding affinity (Claveria-Gimeno et al., 2017).

9.4.7 Peptide arginase inhibitory activities

Five synthesized peptides (CQR, DR, DQR, QER, REGNH) were evaluated against arginase I, yielding an experimental activity hierarchy of QER (18% inhibition at $100\text{ }\mu\text{g/mL}$) $\approx$ REGNH (17%) $\approx$ DQR (16%) $\gg$ DR ($<2\%$) $\approx$ CQR (0%), with 3 of 5 tested peptides showing measurable inhibitory activity. However, given that only 5 of 24 candidate peptides were experimentally evaluated, the generalization of this observation to the broader candidate pool remains uncertain. The failure of DR, the top-ranked peptide in both target prediction and docking, and CQR's complete inactivity highlight the limitations of current *in silico* methods for predicting functional enzyme inhibition. Despite possessing theoretically optimal features (aspartate carboxylate for manganese coordination, C-terminal arginine for electrostatic interactions, minimal steric bulk, highest predicted binding affinity), DR exhibited negligible activity ($<2\%$ inhibition even at $100\text{ }\mu\text{g/mL}$). This disconnect between binding prediction and functional activity has been observed in other metalloenzyme systems (Saini et al., 2021). DR's compact dipeptide structure may lack sufficient contact points to achieve the residence time required for effective inhibition, and MD simulations suggest a possible preferential stability toward arginase II over arginase I — though

this remains experimentally unverified. CQR's similar computational preference for arginase II, coupled with its inactivity toward arginase I, supports this hypothesis.

QER's experimental success despite its lower computational ranking (15th in target prediction, 2nd in docking) is notable and suggests that the extended tripeptide structure provides advantages not captured by scoring functions. The glutamine-glutamate-arginine sequence may enable simultaneous engagement of multiple active-site residues, thereby prolonging residence time relative to the shorter DR dipeptide. The glutamate residue could form critical hydrogen bonds with active site His126 or Asn130, residues known to participate in substrate recognition and orientation (Kuramitsu et al., 2023; Mortier et al., 2017). This multi-point attachment mechanism would explain why QER achieves functional inhibition despite potentially lower intrinsic binding affinity than DR at any single site. The results suggest that it is the synergy of multiple weak-to-moderate interactions, rather than a single strong interaction, that produces effective catalytic disruption by QER (Tommasi et al., 2018). Comparison of DR (inactive), DQR (moderate activity, $K_i = 150 \mu\text{M}$), and QER (highest activity, $K_i = 330 \mu\text{M}$) indicates that effective arginase inhibition requires not merely strong binding but strategic positioning of functional groups to disrupt catalysis. The observation that DQR has a lower K_i yet lower percent inhibition than QER further illustrates the complex relationship between binding affinity and functional inhibition.

9.4.8 Kinetic Mechanisms reveal complex peptide-arginase interactions

Enzyme kinetic analysis of DQR, QER, and REGNH revealed a consistent pattern of increased apparent K_m and decreased V_{max} in the presence of peptide inhibitors (Table 9.4, Figure 11B–D), consistent with an apparent mixed-type inhibition pattern in which the peptides may interact with both the free enzyme and the enzyme-substrate complex. Kinetic parameters were derived from direct nonlinear regression fitting to the Michaelis-Menten model, with Lineweaver-Burk

plots used to visualize the inhibition pattern. DQR exhibited the lowest K_i (150 μM), indicating the highest binding affinity among the three peptides tested. QER displayed a higher K_i (330 μM) despite achieving the highest percent inhibition (~18%), further illustrating the complex relationship between binding affinity and functional inhibition. Although QER differs from DQR by only a single residue substitution (Asp→Glu at position 2), this difference in the positioning of the acidic side chain appears to influence inhibitory efficacy, consistent with studies demonstrating that minor modifications in inhibitor geometry can substantially affect potency (Di Costanzo et al., 2005).

The apparent mixed-type inhibition observed for all three peptides contrasts with the non-competitive allosteric mechanism previously reported for REGNH (Gao et al., 2021). This discrepancy may result from differences in the enzyme source (recombinant human arginase versus bovine liver arginase). REGNH displayed kinetic parameters (K_i ~320 μM) and functional inhibition (17%) comparable to those of the lupin-derived peptides, confirming that the computational pipeline successfully identified peptides with activity comparable to the only known peptide-based arginase inhibitor. The modest inhibitory activities observed should be interpreted in the context of the assay conditions. The 100 mM L-arginine substrate concentration far exceeds physiological levels (100–200 μM), which substantially reduces the apparent efficacy of competitive and mixed-type inhibitors. Additionally, the maximum inhibition did not reach 50%, precluding IC_{50} determination; K_i values therefore provide a more informative measure of inhibitory potency for these peptides.

4.9 Methodological limitations and future directions

Several methodological limitations should be acknowledged. The *in silico* enzymatic hydrolysis using BIOPEP-UWM assumes ideal cleavage at all predicted sites without accounting for protein

tertiary structure, kinetic competition between proteases, or sequential gastric-intestinal pH transitions. Consequently, the predicted peptide profiles may differ from those generated under physiological digestion conditions (Minkiewicz et al., 2019). ADME predictions from SwissADME were developed for small organic molecules, and their reliability for short charged peptides remains uncertain. The Similarity Ensemble Approach (SEA) failed to predict activity for the known inhibitor REGNH (Gao et al., 2021), and poor correlation was observed between computational rankings and experimental outcomes, confirming the limited applicability of these tools to peptide-based enzyme inhibitors. Molecular docking employed rigid receptor structures (PDB: 2AEB and 4IXU) that do not capture arginase active site plasticity (Mortier et al., 2017), and AutoDock Vina's scoring function does not explicitly model coordination interactions with the binuclear manganese cluster. Conventional docking validation (crystal ligand redocking, enrichment tests) was not performed due to the absence of curated peptide-specific decoy datasets; validation relied on a comparative strategy using known inhibitors under identical conditions. Molecular dynamics simulations were performed as single 100 ns trajectories without replicates, using the OPLS-2005 force field, which was not specifically parameterized for short charged peptides. The computational observations, therefore, represent preliminary indicators of relative binding stability rather than definitive mechanistic conclusions.

Experimental validation was restricted to bovine liver arginase I, which may differ from human recombinant arginase in kinetic behavior and inhibitor sensitivity. Human recombinant arginase II was not commercially available, leaving the computationally predicted isoform-specific interactions experimentally unverified. Only 5 of the 24 candidate peptides were synthesized and tested due to cost constraints, and the observed inhibitory activity was modest (~17% maximum inhibition at 100 $\mu\text{g/mL}$), insufficient for IC_{50} determination. No experimental assessment of

peptide stability, gastrointestinal survival, membrane permeability, or bioavailability was conducted. The translation of *in vitro* enzyme inhibition at supraphysiological conditions to any physiological relevance remains entirely undemonstrated and warrants investigation using Caco-2 transport assays, simulated gastrointestinal digestion stability studies, and ultimately *in vivo* models. Finally, the REGNH scaffold-based design strategy inherently constrained the chemical diversity that could be explored. Alternative approaches, such as high-throughput hydrolysate screening or machine learning-guided peptide design, may identify more potent and structurally diverse arginase-modulatory peptides.

9.5 CONCLUSIONS

This study employed an integrated computational-experimental approach to identify and characterize arginase-modulatory peptides from white lupin (*L. albus*) proteins. Through rational design based on the REGNH scaffold, *in silico* proteolysis, target prediction, molecular docking, and molecular dynamics simulations, 24 C-terminal arginine-containing candidate peptides were identified. Of the five peptides synthesized and evaluated against arginase I, three (DQR, QER, REGNH) showed modest but reproducible inhibitory activity (~16–18% at 100 µg/mL), with DQR exhibiting the lowest K_i (150 µM) and an apparent mixed-type inhibition pattern. Notable discrepancies between computational predictions and experimental outcomes, particularly DR's inactivity despite top computational ranking, demonstrate that binding affinity alone does not reliably predict functional enzyme inhibition. The inhibitory activities observed were modest under the assay conditions used, and the translation of these findings to physiologically relevant contexts remains to be established through assessments of peptide stability, bioavailability studies, and testing with human recombinant arginase isoforms. These findings represent a preliminary characterization of the arginase-modulatory potential of white lupin protein-derived peptides and

provide structure-activity insights that may inform future efforts to identify more potent enzyme-modulatory peptides from food protein sources.

CRedit AUTHORSHIP CONTRIBUTION STATEMENT

Stanley Chukwuejim: Writing – original draft, Investigation, Methodology, Formal analysis.

Toluwase Hezekiah Fatoki: Methodology, Formal analysis, Writing – review & editing.

Rotimi Emmanuel Aluko: Writing – review & editing, Validation, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

DECLARATION OF COMPETING INTEREST

The author is an Editorial Board Member/Editor-in-Chief/Associate Editor/Guest Editor for Journal of Functional Foods, Food Chemistry, and Food Chemistry X and was not involved in the editorial review or the decision to publish this article. S.C. and T.H.F. declare no conflict of interest.

DATA AVAILABILITY

Data will be made available on request.

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CHAPTER TEN

GENERAL DISCUSSION AND CONCLUSIONS

10.1 Summary

This thesis aimed to advance the utilization of lupin seed proteins as versatile, sustainable food ingredients through two complementary research streams. The first stream systematically characterized the physicochemical, structural, and techno-functional properties of white lupin (*Lupinus albus*) and blue lupin (*Lupinus angustifolius*) proteins at progressive levels of fractionation, from whole isolates through solubility-based fractions (albumin, globulin, glutelin) to sedimentation-based fractions (vicilin and legumin), and investigated transglutaminase-catalyzed cross-linking as a strategy for enhancing emulsifying performance. The second stream explored the generation of bioactive peptides from lupin protein hydrolysates and employed both *in vitro* and *in silico* rational design to identify, characterize, and validate peptides with arginase-inhibitory activity. Together, these two streams provide an integrated understanding of lupin proteins as both functional food ingredients and precursors to health-promoting bioactive peptides.

10.2 Synthesis of Key Findings and Mechanistic Insights

10.2.1 Structure-function relationships across species and fractionation levels

A central outcome of this research is the demonstration that the techno-functional properties of lupin proteins are strongly governed by their structural organization across multiple hierarchical levels, from whole isolates to individual storage protein fractions. The results showed that functional behavior is not an intrinsic property of the protein source alone but rather emerges from the combined contributions of structurally distinct components. At the isolate level, both white and blue lupin proteins exhibited functional properties comparable to those of soybean protein isolate

under similar experimental conditions, particularly in emulsification. However, these properties were highly dependent on environmental factors such as pH and protein concentration. Reduced performance near the isoelectric point highlighted the importance of electrostatic interactions in maintaining solubility and interfacial stability, while improved functionality at neutral and alkaline pH reflected enhanced molecular dispersion and interfacial activity.

Fractionation of protein isolates into albumin, globulin, and glutelin classes revealed that isolate-level behavior masks important differences among individual protein components. White lupin was characterized by a higher proportion of globulins, whereas blue lupin exhibited a more balanced distribution between globulin and glutelin fractions. Blue lupin fractions generally displayed higher intrinsic fluorescence intensities, suggesting greater exposure of aromatic residues and a more flexible conformation that facilitates interfacial adsorption. Notably, the globulin fraction of white lupin was uniquely not limited in sulfur-containing amino acids, a nutritional advantage absent from the whole isolate, indicating that fractionation can selectively enrich protein components with more favorable compositional quality.

Further separation of the globulin fraction into vicilin (7S) and legumin (11S) sub-fractions provided additional mechanistic insight. Vicilin fractions lacking interchain disulfide bonds exhibited greater molecular flexibility and surface activity, thereby enhancing emulsifying and foaming properties over a broader pH range. Legumin fractions, in contrast, exhibited higher thermal stability and lower gelation concentrations, reflecting their more compact, disulphide-stabilized hexameric quaternary structure. These contrasting structural architectures confirm that protein functionality is closely linked to molecular organization and that the balance between flexibility and stability determines suitability for specific food applications.

10.2.2 Influence of pH and enzymatic treatment on functionality

Across Chapters 3–7, pH emerged as the most influential environmental variable governing the techno-functional behaviour of lupin proteins. Functional performance was consistently reduced near the isoelectric point (pH 4–5), where diminished net charge limits electrostatic repulsion and promotes protein aggregation, thereby reducing effective interfacial adsorption. Structural analysis provided a mechanistic basis for these observations. At acidic pH near the isoelectric point, protein isolates exhibited increased β -sheet content, which is generally associated with more ordered and less flexible conformations. In contrast, at neutral and alkaline pH, an increase in α -helical content was observed, coinciding with improved emulsifying performance, including smaller droplet sizes and enhanced stability. This correlation between higher α -helix to β -sheet ratios and superior emulsification represents a useful predictive structural marker: conformations that favor amphiphilic, flexible chain arrangements at the interface translate into more effective interfacial film formation.

Treatment with microbial transglutaminase resulted in the formation of higher-molecular-weight protein polymers via covalent ϵ -(γ -glutamyl)lysine linkages, with moderate enzyme concentrations (10–15 U/g protein) providing the most favorable balance between polymerization and functionality. Under these conditions, cross-linked proteins exhibited smaller droplet sizes, stronger interfacial films, and the development of continuous-phase protein networks that contributed to both interfacial and bulk-phase stabilization. The most notable functional improvement occurred under acidic conditions (pH 3), where native proteins typically perform poorly due to limited steric stabilization despite high net charge; cross-linking addressed this limitation by creating larger amphiphilic polymers that anchor more effectively at the interface. Importantly, excessive cross-linking at higher enzyme concentrations proved counterproductive,

as the formation of extensively aggregated structures confirmed by SDS-PAGE compromised solubility and interfacial mobility, underscoring the need to optimize the extent of modification for each target application.

10.2.3 Discovery and characterization of arginase-inhibitory peptides

The second research stream extended the value of lupin proteins beyond their functional role in food systems to their potential as sources of bioactive peptides. Enzymatic hydrolysis of lupin protein isolates, followed by chromatographic fractionation and mass spectrometric analysis, demonstrated that lupin proteins can serve as a source of peptides with arginase-inhibitory activity. Multiple peptide sequences were identified from the most active fractions, including the short hydrophobic hexapeptide DMGAVA and the longer polycationic 16-mer VVALKKVGVLKKPGPT. Detailed inhibition kinetics and computational analyses revealed that these peptides operate via mixed-type inhibition through complementary molecular strategies: differences in peptide length, charge distribution, and hydrophobicity influence binding behavior and determine whether the peptide engages the active-site cavity directly or interacts with an extended binding surface involving peripheral residues. An important species-specific observation was that white lupin hydrolysates consistently exhibited more potent arginase-inhibitory activity than their blue lupin counterparts, likely reflecting the distinct conglutin ratios and amino acid compositions of the two species that generate a peptide pool inherently richer in sequences capable of interacting with the arginase active site.

Complementing the experimental approach, a computation-first rational design strategy based on the REGNH scaffold and white lupin genome mining identified the tripeptides DQR and QER as additional lead compounds that achieve arginase inhibition through an apparent mixed-type mechanism. Importantly, discrepancies were observed between computational binding affinity

predictions and experimentally measured inhibitory activity—most notably, DR ranked highest computationally but showed negligible *in vitro* activity, while QER succeeded despite a lower computational ranking. These findings reveal that binding affinity alone does not reliably predict functional enzyme inhibition, as effective modulation requires not only stable binding but also productive disruption of catalytic geometry. This methodological insight has broad implications for the field of bioactive peptide discovery, emphasizing the indispensability of experimental validation alongside computational screening.

10.3 Contribution to Knowledge

This thesis makes the following original contributions to scientific literature:

1. It provides a comprehensive side-by-side characterization of white and blue lupin proteins, spanning three levels of fractionation (whole isolate, Osborne fractions, and vicilin/legumin fractions) with consistent analytical methods applied across all levels and both species. To the best of our knowledge, no prior study has systematically compared these two commercially important lupin species at this depth of structural and functional resolution.
2. It establishes a quantitative relationship between secondary structure composition and emulsifying performance in lupin proteins. The circular dichroism data demonstrated that higher α -helix-to- β -sheet ratios correlate with smaller oil droplet sizes and improved emulsion stability, providing a predictive structural marker of emulsifying capacity to guide ingredient selection and process optimization.
3. It extends the application of transglutaminase-catalyzed cross-linking to lupin proteins, establishing the dose–response relationship between enzyme concentration, secondary structure changes, and emulsifying performance across a range of pH conditions. The identification of 10–

15 U/g as the optimal enzyme concentration window provides directly actionable guidance for ingredient manufacturers.

4. It pioneers the identification of arginase-inhibitory peptides from lupin proteins. The characterization of DMGAVA, VVALKKVGVLKKPGPT, QER, and DQR, including their kinetic properties, inhibitory mechanisms, and binding interactions, establishes a molecular foundation for the development of lupin-based functional foods targeting arginase-related disorders.

5. It integrates wet-laboratory enzymology with *in silico* rational design, demonstrating an avenue for bioactive peptide discovery from plant proteins while critically evaluating the limitations of predictive computational methods.

10.4 Limitations of the Study

While this thesis provides a comprehensive investigation of lupin protein functionality and bioactivity, several limitations should be acknowledged. The functional property studies were conducted under controlled laboratory conditions using model emulsions and foam systems. Real food matrices contain additional components, such as salts, sugars, polysaccharides, lipids of varying composition, and competing surfactants that may interact with lupin proteins and alter their functional performance in ways not captured by the model systems employed here. Also, emulsion stability was evaluated over relatively short time frames (30 minutes), which, while useful for initial characterization and comparative ranking, does not reflect the long-term storage stability (weeks to months) required for commercial food products. Accelerated shelf-life testing and storage studies under realistic temperature and light conditions would be needed to validate the commercial viability of lupin protein-stabilized emulsions. The *in vitro* protein digestibility assay (multi-enzyme pH drop method) does not account for *in vivo* factors such as gastric emptying

rates, interactions with the gut microbiota, individual physiological variation, and food matrix effects on protein digestibility. More physiologically relevant digestibility models, such as the INFOGEST static or dynamic *in vitro* digestion protocols, would provide a more detailed assessment of the nutritional quality of lupin protein isolates and fractions.

The arginase-inhibitory activities of the identified peptides were assessed exclusively *in vitro* using purified enzyme. The bioavailability of these peptides, including their stability during gastrointestinal digestion, absorption across the intestinal epithelium, resistance to serum peptidases, and ability to reach target tissues at effective concentrations, remains uncharacterized. The ADME predictions generated in Chapter 9 suggested low gastrointestinal absorption for most candidate peptides, which may limit their therapeutic potential when administered orally. The study focused exclusively on two lupin species (*L. albus* and *L. angustifolius*) and did not include other cultivated species such as *L. luteus* (yellow lupin) or *L. mutabilis* (Andean lupin), which may offer different protein profiles and functional characteristics. Additionally, cultivar-to-cultivar variation within species was not examined, and the results obtained may not be fully generalizable across all commercial cultivars of white and blue lupin. Finally, the discrepancies between computational binding affinity predictions and experimental arginase-inhibitory activity highlight inherent limitations of current molecular docking and dynamics approaches in predicting functional enzyme inhibition. Binding affinity is a necessary but not sufficient condition for inhibition, and the computational methods employed could not fully account for factors such as peptide behavior in solution, entropic contributions, and the dynamic nature of the enzyme active site under assay conditions.

10.5 Proposed future studies

Several avenues of future research emerge from this thesis. In the area of techno-functional characterization, studies should evaluate the performance of lupin protein fractions in actual food systems, including plant-based beverages, yogurt alternatives, salad dressings, and meat analogues under realistic formulation and processing conditions. Extended shelf-life studies incorporating accelerated storage tests, thermal processing, and freeze–thaw cycling would be essential for establishing commercial viability. The interactions of lupin proteins with other food matrix components, such as polysaccharides, lipids, and mineral salts, should be systematically investigated to understand synergistic or antagonistic effects on functionality.

In the bioactive peptide domain, the most pressing need is to establish the physiological relevance of the identified arginase-inhibitory peptides through cell-based assays (e.g., endothelial cell NO production, T-cell proliferation in immune models) and preclinical animal studies. Bioavailability studies using Caco-2 cell monolayers and *in vivo* pharmacokinetic models should be conducted to determine whether the peptides survive gastrointestinal digestion, cross the intestinal barrier, and reach target tissues at effective concentrations. Structure-guided optimization of lead peptides, particularly QER, DQR, and DMGAVA, through strategic amino acid substitutions and additions informed by the molecular docking and dynamics data generated in this thesis could yield more potent analogues with improved stability and bioavailability. Additionally, the *in silico* pipeline developed in Chapter 9 should be extended to screen for lupin-derived peptides with other bioactivities, such as inhibition of angiotensin-converting enzyme, Renin, dipeptidyl peptidase-IV, and α -glucosidase, which are relevant to hypertension, diabetes, and high blood pressure. The white lupin genome database used in this work provides a rich resource for mining additional bioactive sequences beyond those associated with arginase inhibition. Finally, pilot-scale

fractionation studies with systematic optimization of processing parameters, including extraction time, dialysis conditions, and centrifugation protocols, would be essential for scaling up the production of specific lupin protein fractions with consistent quality and functionality.

An important next step is to evaluate the economic feasibility and industrial scalability of the approaches developed in this study. The investigated technologies vary considerably in their readiness for industrial scale-up. Transglutaminase cross-linking employs a food-grade microbial enzyme already widely used in the food industry, and enzymatic hydrolysis is also readily scalable. In contrast, the multi-step fractionation and reverse-phase HPLC purification procedures remain laboratory-scale techniques and would require more commercially viable alternatives, such as membrane-based separation or targeted peptide synthesis, for industrial application. Ultimately, the economic viability of these approaches will depend on whether the value of the recovered protein fractions or bioactive peptides is sufficient to offset the additional processing costs. This is a consideration that is more easily justified for high-value nutraceutical ingredients than for bulk food ingredients. Consequently, comprehensive techno-economic analysis and life-cycle assessment, incorporating raw material costs, process yields, energy requirements, and co-product valorization, will be essential for determining the commercial potential of translating these findings into industrial practice.

10.6 Closing Statements

This thesis demonstrates that lupin seed proteins possess a depth of functional versatility and bioactive potential that has been largely unexplored. By evaluating the potential of two commercially important lupin species across multiple fractionation levels, establishing quantitative structure–function relationships, and pioneering the discovery of arginase-inhibitory peptides from a previously unexplored legume source, this work lays both the scientific foundation

and the practical framework for the expanded utilization of lupin in the global food and nutraceutical industries. As demand for sustainable, plant-based protein ingredients continues to grow, lupin is well-positioned to transition from an underutilized crop to a functional protein source.