EFFECT OF DEFATTING METHOD ON THE STRUCTURE AND FUNCTION OF MORINGA SEED PROTEINS

By

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ABSTRACT

The global drive for sustainable food systems highlights the potential of underutilized crops like *Moringa stenopetala* (MS) for their nutritional and functional properties. This study investigates how various defatting methods influence the physicochemical and functional attributes of MS protein isolates prepared using alkaline isoelectric precipitation (ISO) and NaCl membrane filtration (MEM_NaCl). Protein isolates were derived from cold-pressed MS meal in its undefatted form (UMGPI) and from cold-pressed MS meal defatted using various solvents: acetone (AMGPI), hexane (HMGPI), methanol (MMGPI), 100 % ethanol (EMGPI), 70 % ethanol (7EMGPI), 50 % ethanol (5EMGPI), and water (WMGPI). ISO yielded the highest protein recovery (41.73 – 83.23 %) and content (69.50 – 83.24 %), surpassing MEM_NaCl, which achieved recovery rates of 12.96 – 63.67 % and protein content of 28.21 – 72.83 %. Consequently, ISO-prepared isolates were prioritized for further analysis. UMGPI exhibited the highest yield, while AMGPI displayed superior protein content. MMGPI stood out for its balanced amino acid profile, high essential amino acids (EAA), sulphur containing amino acids (SCAA), and hydrophobic amino acids (HAA), along with the highest surface hydrophobicity (32.67). AMGPI demonstrated exceptional functionality, with solubility of 25 – 75 % across pH levels 3 – 9. It also exhibited the highest foaming capacity (~80 %) and stability at pH 7 and 9. Thermal analysis revealed its stability with an onset temperature (Tθ) of 85.0 ^{o}C, peak temperature (T_p) of 88.77 ^{o}C, and enthalpy change (ΔH) of 0.25 J/g. While solubility peaked at pH 3, this acidic environment limited its application for emulsion formulations, making pH 7 and 9 more suitable due to improved stability and air incorporation. Intrinsic fluorescence analysis revealed a weak tyrosine peak at 303 nm and a pronounced tryptophan peak at 348 nm, with MMGPI showing the highest fluorescence intensity (1000 – 3000) at pH 3. Among ethanol-defatted samples, 7EMGPI exhibited

superior solubility and stable emulsifying properties. It had the highest in vitro protein digestibility (81.32%), and the highest oil-holding capacity at 3.93 g/g, while MMGPI had the lowest (2.98 g/g). Water-holding capacity was highest in 5EMGPI (2.81 g/g) and lowest in UMGPI (2.15 g/g). UMGPI retained the highest total phenolic content at 1.39 – 1.53 mg GAE/g, while WMGPI had the lowest (0.57 – 1.29 mg GAE/g). Bitterness intensities ranged from MMGPI (23.34) to AMGPI (28.24). Enzymatic hydrolysis with alcalase (ALH), flavourzyme (FLH), and pepsin + pancreatin (PPH) further enhanced protein bioactivity. Bioactivity was tested at different concentrations. ALH and PPH consistently outperformed FLH in most of the assays. PPH achieved the highest protein recovery (86.23 %), superoxide scavenging (25.77 – 84.55 %), and metal chelation (4.64 %) ALH demonstrated the highest protein content (72.16 %), lowest peptide molecular weight (0.38 – 2.19 kDa), and potent bioactivities, including ACE inhibition (15.07 – 66.56 %) and renin inhibition (24.89 – 51.68 %). FLH excelled in pancreatic lipase inhibition (42.06 – 64.38 %) and arginase inhibition (13.18 – 34.57 %). This study underscores the importance of defatting methods in optimizing MS protein functionality and bioactivity, with AMGPI and 7EMGPI as promising candidates for functional foods and nutraceuticals. Enzymatic hydrolysis, especially with alcalase, further broadens their application potential.

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FOREWARD

This thesis was prepared using the manuscript format and comprises two main manuscripts, following the general introduction and literature review chapters. Manuscript 1 investigates the impact of solvent type on the structural and functional properties of protein isolates derived from defatted *Moringa stenopetala* seed meal. Manuscript 2 explores the *in vitro* bioactive properties of enzymatic protein hydrolysates from *Moringa stenopetala* seeds. To ensure a seamless progression between chapters, a transition statement is included at the end of Manuscript 1, linking it to the subsequent chapter. The final chapter presents a comprehensive summary of the study along with recommendations for future research directions.

TABLE OF CONTENTS

ABSTRACT ii
FOREWARD v
LIST OF TABLES x
LIST OF FIGURES xii
LIST OF ABBREVIATIONS xv
CHAPTER ONE 1
1.0 INTRODUCTION 1
1.1 Hypotheses of the study 5
1.2 Research objectives 5
REFERENCES 7
CHAPTER TWO 12
2.0 LITERATURE REVIEW 12
2.1 Moringa stenopetala (MS) 12
2.2 Nutritional Composition and Industrial Applications of MS 13
2.3 MS Seeds as Alternative Sources of Protein 16
2.4 Protein Isolation 17
2.4.1 Alkaline extraction coupled with isoelectric pH precipitation (ISO) 18
2.4.2 Salt precipitation (SP) 18
2.4.3 Micellar precipitation (MP) 19
2.4.4 Membrane ultrafiltration (MU) 20
2.5 Defatting Method 21
2.6 Structural-Functional Properties of Food Proteins 22
2.6.1 Solubility properties 24
2.6.3 Emulsifying properties 25
2.6.4 Foaming properties 26
2.6.5 Water holding capacity 27
2.6.6 Oil holding capacity 27
2.7 Bioactive Peptides 32
2.8 Bioactive Properties of Protein Hydrolysates 34
2.8.1 Antioxidant properties 34
2.8.2 Antihypertensive properties 36
2.8.3 Anti-cancer properties 39
2.8.4 Antidiabetic and anti-obesity properties 39

2.9 Bitter Taste in MS 47
REFERENCES 48
CHAPTER THREE 74
3.0 EFFECTS OF SOLVENT TYPE ON THE STRUCTURAL AND FUNCTIONAL PROPERTIES OF PROTEIN ISOLATES OBTAINED FROM DEFATTED *MORINGA STENOPETALA* SEED MEAL 74
3.1 Introduction 75
3.2 Materials and Methods 79
3.2.1 Raw materials and sample preparation (defatting process) 79
3.2.2 Lipid extraction for fatty acid analysis 82
3.2.3 Lipid methylation and gas chromatography 82
3.2.4 Protein isolation using alkaline solubilization followed by pH isoelectric precipitation 83
3.2.5 Protein isolation using NaCl solubilization coupled with membrane isolation 85
3.2.6 Proximate composition 87
3.2.7 Amino acid composition 87
3.2.8 Total polyphenol content 87
3.2.9 E-tongue testing 88
3.2.10 Intrinsic fluorescence 88
3.2.11 Surface hydrophobicity 89
3.2.12 Circular dichroism 89
3.2.13 Differential scanning calorimetry 90
3.2.14 Protein solubility 90
3.2.15 Heat coagulability 91
3.2.16 Emulsion formation and stability 91
3.2.17 Foam formation and stability 92
3.2.18 Water and oil holding capacity 93
3.2.19 In vitro protein digestibility (IVPD) 93
3.2.20 Statistical analysis 94
3.3 Results and Discussion 95
3.3.1 Fatty acid composition MS 95
3.3.2 Protein content and yield of MSPI 101
3.3.3 Proximate composition 105
3.3.4 Amino acid composition 109
3.3.5 Total polyphenol content 117
3.3.6 E-tongue testing 121

3.3.7 Surface hydrophobicity 122
3.3.8 Differential scanning calorimetry 124
3.3.10 In vitro protein digestibility (IVPD) 127
3.3.11 Intrinsic fluorescence 128
3.3.12 Circular dichroism (secondary and tertiary structures) 132
3.3.13 Protein solubility 139
3.3.14 Heat coagulability 141
3.3.15 Emulsion formation & stability 143
3.3.16 Foaming capacity and foam stability 150
3.4 Conclusion 157
REFERENCES 159
TRANSITION STATEMENT 187
CHAPTER FOUR 188
4.0 IN VITRO BIOACTIVE PROPERTIES OF *MORINGA STENOPETALA* SEED ENZYMATIC PROTEIN HYDROLYSATES 188
4.1 Introduction 189
4.2 Materials and Methods 191
4.2.1 Raw materials and sample preparation 191
4.2.2 Protein hydrolysis 191
4.2.3 Protein content 194
4.2.4 Amino acid composition 194
4.2.5 Molecular weight distribution 195
4.2.6 Surface hydrophobicity 195
4.2.8 Renin inhibition 196
4.2.9 Arginase inhibition 197
4.2.10 α-amylase inhibition 197
4.2.11 α-glucosidase inhibition 198
4.2.12 Pancreatic lipase inhibition 199
4.2.13 Trypsin inhibition 200
4.2.13 Chymotrypsin inhibition 200
4.2.14 Acetylcholinesterase (AChE) inhibition 201
4.2.15 DPPH radical scavenging activity (DRSA) 202
4.2.16 Superoxide radical scavenging activity (SRSA) 202
4.2.17 Hydroxyl radical scavenging activity (HRSA) 203
4.2.18 Ferric reducing antioxidant power (FRAP) 203

4.2.19 Metal chelation activity (MCA) 204
4.2.20 Inhibition of linoleic acid peroxidation 204
4.2.21 Statistical analysis 205
4.3 Results and discussion 205
4.3.1 Hydrolyzed Protein yield and protein content of MSPH 205
4.3.2 Surface hydrophobicity of MSPH 208
4.3.3 Amino acid composition of MSPH 209
4.3.4 Molecular weight distribution of MSPH 213
4.3.5 Angiotensin converting enzyme (ACE) inhibitory activity 216
4.3.6 Renin-inhibitory activity 217
4.3.7 Arginase inhibitory activity of MSPH 219
4.3.8 α-amylase inhibitory activity 222
4.3.9 α-glucosidase inhibitory activity 222
4.3.10 Pancreatic lipase inhibitory activity 223
4.3.11 Trypsin inhibitory activity of MSPH 226
4.3.12 Chymotrypsin inhibitory activity 227
4.3.13 Acetylcholinesterase inhibitory 228
4.3.14 DPPH radical scavenging activity (DRSA) 231
4.3.15 Superoxide radical scavenging activity (SRSA) 232
4.3.16 Hydroxyl radical scavenging activity (HRSA) 233
4.3.17 Ferric reducing antioxidant power (FRAP) 235
4.3.18 Metal chelation activity (MCA) 235
4.3.19 Linoleic acid peroxidation inhibitory activity 236
4.4 Conclusion 241
REFERENCES 242
CHAPTER FIVE 262
5.0 GENERAL SUMMARY AND CONCLUSIONS 262
5.1 General Summary 262
5.2 Significance of Research 263
5.3 Future Directions 264

LIST OF TABLES

Chapter Two

Table 2.1: Effects of protein extraction methods on Functional properties of plant-based protein isolates 30

Table 2.2: Bioactive properties of plant-based protein hydrolysates 44

Chapter Three

Table 3.1: Fatty acid composition of oil extracts from undefatted MS meal (UMG) or meal defatted with; acetone (AMG), 100% ethanol (EMG), and hexane (HMG). 98

Table 3.2: Protein content (%) and yield (%) of protein isolates obtained from undefatted MS meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI)..104

Table 3.3: Proximate composition (%) of protein isolates obtained from undefatted MS meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI)..108

Table 3.4: Amino acid composition (%) of protein isolates obtained from undefatted Moringa stenopetala meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI). 113

Table 3.5: Functional and thermal properties of protein isolates obtained from undefatted Moringa stenopetala meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI). 119

Table 3.6: Secondary structure fractions of protein isolates obtained from undefatted MS meal (UMGPI), or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI)..137

Chapter Four

Table 4 1: Protein content, protein yield, and surface hydrophobicity of protein hydrolysate obtained from MS meal hydrolyzed with alcalase (ALH), flavourzyme (FLH), and pepsin + pancreatin (PPH).....207

Table 4 2: Amino acid composition of protein hydrolysate obtained from MS meal hydrolyzed with alcalase (ALH), flavourzyme (FLH), and pepsin + pancreatin (PPH).....211

LIST OF FIGURES

Chapter Two

Figure 2. 1: Renin-Angiotensin System for blood pressure regulation.....38

Chapter Three

Figure 3. 1: Defatting process of MS seed meal.....81

Figure 3. 2: Isolation of MS seed proteins by alkaline solubilization followed by isoelectric pH precipitation. 84

Figure 3. 3: Isolation of MS seed protein (NaCl membrane ultrafiltration).....86

Figure 3. 4: Intrinsic fluorescence of protein isolates obtained from undefatted MS meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI).....131

Figure 3. 5: Near UV of protein isolates obtained from undefatted MS meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI). 136

Figure 3. 6: Protein solubility (A) and heat coagulability (B) of protein isolates obtained from undefatted MS meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI). 142

Figure 3. 7: Oil droplet size of emulsions formed by protein isolates obtained from undefatted MS meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI).....147

Figure 3. 8: Stability of emulsions formed with protein isolates obtained from undefatted MS meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI)..149

Figure 3. 9: Foaming capacity of protein isolates obtained from undefatted MS meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI). 154
Figure 3. 10: Stability of foams produced by protein isolates obtained from undefatted MS meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI)..156

Chapter Four

Figure 4. 1: Enzymatic hydrolysis of MSPI. Enzymes used and optimal conditions (Alcalase: pH 8.0; 50°C, Flavourzyme: pH 6.5; 50°C, Pepsin (pH 2.0) + pancreatin (pH 7.5); 37°C). 193
Figure 4. 2: Molecular weight distribution of MSPH. ALH – Alcalase hydrolysate, PPH – Pepsin + pancreatin hydrolysate, FLH – Flavourzyme hydrolysate. 215
Figure 4. 3: ACE (A), renin (B), and arginase (C) inhibitory activity of MSPH. ALH – Alcalase hydrolysate, PPH – Pepsin + pancreatin hydrolysate, FLH – Flavourzyme hydrolysate. Each value represents the mean ± standard deviation from three independent measurements. Different superscript letters denote significant differences ($p < 0.05$).....221
Figure 4. 4: α-amylase (A), α-glucosidase (B), and pancreatic lipase (C) inhibitory activity of MSPH. ALH – Alcalase hydrolysate, PPH – Pepsin + pancreatin hydrolysate, FLH – Flavourzyme hydrolysate. Each value represents the mean ± standard deviation from three independent measurements. Different superscript letters denote significant differences ($p < 0.05$).....225
Figure 4. 5: Trypsin (A), chymotrypsin (B), and AChE (C) inhibitory activity of MSPH. ALH – Alcalase hydrolysate, PPH – Pepsin + pancreatin hydrolysate, FLH – Flavourzyme hydrolysate. Each value represents the mean ± standard deviation from three independent measurements. Different superscript letters denote significant differences ($p < 0.05$). 230

Figure 4. 6: DPPH (A), superoxide (B), and hydroxyl (C) radical scavenging activity of MSPH. ALH – Alcalase hydrolysate, PPH – Pepsin + pancreatin hydrolysate, FLH – Flavourzyme hydrolysate. Each value represents the mean ± standard deviation from three independent measurements. Different superscript letters denote significant differences ($p < 0.05$).....234

Figure 4. 7: Ferric reducing antioxidant power (A), metal chelation activity (B), and linoleic acid peroxidation (C) inhibitory activity of MSPH. ALH – Alcalase hydrolysate, PPH – Pepsin + pancreatin hydrolysate, FLH – Flavourzyme hydrolysate. Each value represents the mean ± standard deviation from three independent measurements. Different superscript letters denote significant differences ($p < 0.05$).....239

LIST OF ABBREVIATIONS

5EMGPI – 50 % ethanol defatted *Moringa stenopetala* meal protein isolate

7EMGPI – 70 % ethanol defatted *Moringa stenopetala* meal protein isolate

8,5M – 80 % methanol and 50 % methanol

8,5M,7A – 80 % methanol, 50 % methanol and 70 % acetone

8M – 80 % methanol

AA – Amino acids

AAA – Aromatic amino acids

ACE – Angiotensin Converting Enzyme

Ala – Alanine

ALH – Alcalase derived *Moringa stenopetala* meal protein hydrolysate

AMGPI – Acetone defatted *Moringa stenopetala* meal protein isolate

ANOVA – Analysis of variance

Arg – Arginine

ASP – Asparagine + Aspartic acid

BCAA – Branched-chain amino acids

BS – Bitterness score

CD – Circular dichroism

Cys – Cysteine

DSC – Differential scanning calorimetry

EAA – Essential amino acids

EAI – Emulsion Activity Index

EMGPI – Ethanol defatted *Moringa stenopetala* meal protein isolate

ESI – Emulsion Stability Index

FC – Foaming capacity

FI – Fluorescence intensity

FLH – Flavourzyme derived *Moringa stenopetala* meal protein hydrolysate

FS – Foam stability

GLU – Glutamine + Glutamic acid

HAA – Hydrophobic amino acids

HCl – Hydrochloric acid

His – Histidine

HMGPI – Hexane defatted *Moringa stenopetala* meal protein isolate

Ile – Isoleucine

ISO – Alkaline Isoelectric precipitation (ISO)

IVPD – In-vitro protein digestibility

Leu – Leucine

Lys – Lysine

MEM_NaCl – Membrane isolated lentil protein using NaCl

Met – Methionine

MMGPI – Methanol defatted *Moringa stenopetala* meal protein isolate

MP – Micellar precipitation (MP)

MS – Moringa stenopetala

MSPH – *Moringa stenopetala* meal protein hydrolysate

MSPI – *Moringa stenopetala* meal protein isolate

MU – Membrane ultrafiltration (MU)

MUFA – Monounsaturated fatty acid

MWCO – Molecular weight cut-off

n-3 – Omega-3 fatty acid

n-6 – Omega-6 fatty acid

NaCl – Sodium chloride

NaOH – Sodium hydroxide

NCAA – Negatively charged amino acids

NEAA – Non-essential amino acids

OHC – Oil holding capacity

PCAA – Positively charged amino acids

Phe – Phenylalanine

pI – Isoelectric point

PPH – Pepsin + pancreatin derived *Moringa stenopetala* meal protein hydrolysate

Pro – Proline

PUFA – Polyunsaturated fatty acid

SCAA – Sulfur-containing amino acids

Ser – Serine

SFA – Saturated fatty acid

SH – Surface hydrophobicity

SP – Salt precipitation (SP)

Thr – Threonine

TPC – Total polyphenol content

Try – Tryptophan

Tyr – Tyrosine

UMGPI – undefatted *Moringa stenopetala* meal

Val – Valine

WHC – Water holding capacity

WMGPI – Water defatted *Moringa stenopetala* meal protein isolate

CHAPTER ONE

1.0 INTRODUCTION

The world's population is growing fast and is projected to hit 9.8 billion by the year 2050 (Modebadze, 2021). Despite this surge, commercial availability of food protein, particularly from plant sources, remains limited to a few crops. Proteins derived from plants are present in a wide range of crops, including beans, nuts, legumes, mushrooms, cereals, and seed meals, which represent a sustainable source of nutrition (Semba et al., 2021). However, many of these plant proteins are underutilized as food protein sources. To address this gap, researchers are increasingly focused on enhancing the use of plant proteins to boost the global food supply and contribute to protein sustainability. Among the plants drawing significant research attention is Moringa.

Moringa is a perennial tropical plant belonging to the monogeneric Moringaceae family (Aderinola et al., 2020). It has become increasingly recognized for its diverse applications across multiple sectors, such as food, healthcare, and cosmetics (Rani & Arumugam, 2017). The Moringa genus consists of 13 species, with *Moringa oleifera* being the most well-known and thoroughly researched (Singh et al., 2020). This species is native to northern India and primarily found in tropical and subtropical areas (Tang et al., 2021). However, other species, such as *Moringa stenopetala* (MS), remain to be less known and underutilized despite their promising potential as food ingredients (Addis et al., 2021).

MS commonly known as African Moringa, holds even greater promise than the popular M. oleifera in terms of its nutritional and functional properties (Hill, 2019). Indigenous to southern Ethiopia, MS is locally known by names such as "Haleko," "Shelagda," or "Shiferaw" (Hagos et al., 2018) and is cultivated across different regions of the country (Seifu, 2015). This overlooked

crop has garnered interest due to its rich nutritional profile and potential health advantages that extend beyond merely providing essential nutrients (Addis et al., 2021). Studies have demonstrated that MS exhibits a range of therapeutic properties, including antihypertensive, antidiabetic, antipyretic, antibacterial, antifungal, hypocholesterolemic, anti-inflammatory, and antiepileptic activities (Alegbeleye, 2018; Aderinola et al., 2019; Soni & Sharma, 2022).

The increasing worldwide demand for functional foods, which offer health advantages beyond mere nutritional value has led to increased consumption of MS leaves, which are now commonly used as additives in both human and animal diets (Stadtlander & Becker, 2017). These leaves have even been processed into food spices (Milla et al., 2021). Beyond the leaves, the entire plant is abundant in essential nutrients including amino acids, vitamin C, and vitamin A, making it a valuable resource for improving diets and promoting better health outcomes (Yessuf, 2020). The seeds of MS, in particular, have garnered considerable attention due to their high nutritional value (Bania et al., 2023). These seeds have been explored for various uses, including oil extraction for cosmetic and pharmaceutical products, as well as fertilizers (Meireles et al., 2020). Moreover, MS seeds have been investigated for their potential in wastewater treatment (Milla et al., 2021). Studies have reported that MS seeds have a greater protein content and a lower fat content when compared to *Moringa oleifera* seeds (Seifu, 2015). Furthermore, they are noted for their improved protein quality, including higher protein digestibility and a superior amino acid profile (Seifu, 2015). These characteristics underscore the potential of MS seeds as a sustainable and nutrient-dense source of plant protein, which could significantly contribute to tackling global food security issues (Abdullahi 2023).

Various research efforts have highlighted the significance of extracting proteins from plants, as doing so can enhance their functional and structural properties when compared to using

whole seed flour, which typically contain high levels of non-protein components (Ma et al., 2022). Protein isolates are particularly valuable because they can serve as versatile ingredients in food formulations (Akharume et al., 2021). The functional characteristics of protein isolates, such as solubility, emulsification, and gelation properties, make them ideal for use in various food products (Aluko, 2012). However, these functional properties are largely influenced by the protein's structure, meaning that any alteration could significantly impact its performance in food applications (Akharume et al., 2021). Previous research has demonstrated that the methods used in protein isolation significantly impact both the function and structure of the resulting proteins (Zhang et al., 2019). One critical factor in this process is the method of defatting, which removes fats from plant materials as a preliminary step before isolating proteins. Defatting can be achieved using solvents or mechanical means, such as cold pressing. However, this step can also disrupt the protein's structure. When the protein structure is compromised during defatting, the protein loses its native conformation, which can lead to a reduction in functionality (Fang et al., 2023). As a result, the protein becomes less effective in its role as a functional ingredient in food formulations because of the negative effects on solubility, emulsifying properties, and other critical functions. During the defatting process, various solvents can be used to extract fat from food samples. These solvents are generally classified into two main types: polar solvents, which include substances like water, methanol, and ethanol, and non-polar solvents, such as hexane, benzene, and pentane (Cravotto et al., 2022). The selection of a solvent significantly impacts the protein structure, as each solvent type exerts different levels of stress during fat extraction (Kumar et al., 2021). Consequently, it is crucial to carefully evaluate defatting methods to understand their specific effects on both the structure and functionality of extracted proteins.

In plant proteins, the sequence of amino acids typically remains in a dormant or inactive state within the protein's primary structure until enzymatic digestion occurs (Xue et al., 2021). This digestion process, catalyzed by specific enzymes, breaks down the protein, releasing shorter peptide chains from the larger structure (Cruz-Casas et al., 2021). These newly released peptide sequences, collectively known as protein hydrolysates, are crucial because they allow the isolation and characterization of peptides based on their sizes and amino acid compositions (Aluko, 2015). In the case of MS seed proteins, enzymatic hydrolysis can release bioactive peptides called short sequences of amino acids with specific biological functions. These bioactive peptides hold significant potential in nutraceutical applications, especially in the management of chronic conditions like diabetes, high blood pressure, and heart disease, among others (Kadam et al., 2024). The bioactive properties of these peptides are largely determined by their specific combination of amino acids that make up the peptide chain (Nwachukwu & Aluko, 2021). These structural elements influence how effectively the peptides interact with biological systems, thus determining their strength in health-related applications (Cunha & Pintado, 2022). Additionally, the specific enzyme employed during protein hydrolysis significantly influences the biological activities of the resulting peptides (Habinshuti et al., 2023). Different proteases cleave proteins at specific points in the amino acid sequence, producing peptides with varying lengths and sequences (Song et al., 2019). These variations can influence the peptides' bioactive properties, such as their ability to act as antioxidants, regulate blood pressure, or modulate immune responses (Aluko, 2012).

Proteins from MS seeds could significantly contribute to tackling worldwide issues such as food insecurity, malnutrition, and poverty, particularly considering the rapidly growing world population. These proteins can be incorporated into food formulations to enhance nutritional content, while their bioactive peptides hold promise for use in nutraceuticals that offer health

benefits beyond basic nutrition. However, in contrast to the well-studied *Moringa oleifera*, the proteins from MS seeds remain largely unexplored. Currently, there is a scarcity of scientific studies exploring the potential applications of MS seed protein hydrolysates in treating and managing chronic diseases. This research seeks to address the existing gap by examining the structural, functional, and bioactive properties of these proteins and their potential therapeutic applications.

1.1 Hypotheses of the study

The research hypothesis posits that treating MS seed with non-polar solvents will result in a more effective fat removal process while preserving the protein's structure and functionality to a greater extent than polar solvents. Additionally, it is anticipated that peptides derived from MS seeds using alcalase will exhibit enhanced bioactive properties.

1.2 Research objectives

The main goal of this study is to investigate how different defatting solvents impact the structural and functional properties of MS seed proteins, as well as to evaluate the bioactive properties of their resulting hydrolysates.

The specific aims of this study are to determine:

1. The relative effectiveness of polar and non-polar solvents for the defatting of MS seed meal.
2. The impact of various defatting solvents on the physicochemical and functional properties of isolated MS seed proteins.

3. The effect of proteases type on enzyme inhibitory and antioxidant properties of MS seed protein hydrolysates.

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Moringa stenopetala* (MS)

Moringa stenopetala (MS), a species within the fast-growing Moringa genus, is widely cultivated in tropical regions (Seifu, 2015). This perennial softwood, part of the Moringaceae family, comprises 13 species with distinct morphological and floral variations. Key species in the genus include *M. concanensis, M. oleifera, M. arborea, M. ruspoliana, M. rivae, M. drouhardii, M. peregrina, M. stenopetala, M. hildebrandtii, M. ovalifolia, M. longituba, M. pygmaea,* and *M. borziana* (Boopathi & Abubakar, 2021). *M. oleifera* has been extensively researched and is widely spread, whereas other species are more regionally specific and are primarily used in local applications (Alhassan et al., 2022). MS, though less studied, shows significant potential for various uses.

MS is native to southern Ethiopia and widely grows across East Africa, widely prevalent in regions like Dherashe, Gamo-Gofa, Kaffa, Konso, Bale, Sidama, Burji, Amaro, and Borena (Nweze & Eke, 2018; Sefiu, 2015). It thrives in sandy, well-drained soil and is notably drought-resistant and climate-resilient, making it suitable for both arid and wet environments (Bania, 2023). Locally referred to as the "cabbage tree," MS is valued for its rapid growth, rich nutritional profile, and medicinal potential (Heredia & Gutierrez-Grijalva, 2021; Adugna et al., 2016). It significantly contributes to food security in dry regions, as MS can live for over a century, with continuous leaf and flower production (Bania, 2023). It grows quickly, reaching about 3 meters within its first year and up to 12 meters, occasionally 15 meters (Nathaniel, 2021). Reproductive maturity is achieved around 2.5 years after which the tree flowers and bears fruit year-round, serving as a consistent

food source (Heredia & Gutierrez-Grijalva, 2021). The pods are long, measuring 19.7 to 50 cm in length and 1.8 to 4 cm in width, initially appearing as twisted bright green structures that straighten upon maturation (Boopathi & Abubakar, 2021). The trunk is thick at the base, often forked, with smooth whitish-gray bark (Santos, 2013). MS has a sprawling crown with many branches, densely pubescent young shoots, and light green compound leaves (Sefiu, 2015). The bi- or tripinnate leaves have elliptical to ovate leaflets, 3.5 – 6.5 cm long and 2 – 3.5 cm wide, with an acute tip and extrafloral nectaries at the base (Boopathi & Abubakar, 2021).

The tree's inflorescence forms a dense, fragrant panicle reaching up to 60 cm. Its flowers are bisexual, radially symmetrical, and pentamerous, with cream or pink sepals and pale-yellow to greenish-yellow petals (Abay et al., 2015). Each flower contains five stamens and multiple staminodes and the superior ovary elongates into a silky cylindrical style topped with a stigmatic lobe (Boopathi & Abubakar, 2021). The fruit consists of long, initially twisting pods that turn reddish as they mature, eventually splitting open to release up to 20 seeds (Dharani & Yenesew, 2022). The seeds are oblong to triangular, with a cream-colored husk and three prominent wings, measuring 2.5–3.5 cm long and 1.5–2 cm wide (Seifu, 2015). All parts of MS are nutrient-rich and offer considerable industrial potential.

2.2 Nutritional Composition and Industrial Applications of MS

MS is a versatile tree widely cultivated for both human consumption and animal feed and known for its exceptional nutritional and medicinal properties. Every part of the tree contains unique compounds, enhancing its value in food, traditional medicine, and nutraceutical applications, making it a readily accessible dietary supplement (Tefera, 2023).

- Roots, Bark, Flowers, and Pods: The roots of MS are traditionally used for their medicinal properties, offering anti-inflammatory and diuretic effects, though caution is advised due to potential toxicity at high doses (Thakur et al., 2024). The bark exhibits antimicrobial properties and is commonly used for wound healing and immune support (Seifu, 2015). MS flowers, rich in essential amino acids and bioactive compounds, are often brewed in teas for their aphrodisiac and anti-inflammatory benefits (Kumar et al., 2021). The pods, typically consumed when young and tender, provide vitamin C, dietary fiber, and antioxidants, commonly incorporated into local dishes (Nathaniel, 2021).
- Leaves: MS leaves are highly nutritious, offering proteins, essential amino acids, vitamins (A, B, C, and K), minerals (potassium, calcium, and iron), and antioxidants (Tissot et al., 2023). They contain bioactive compounds, such as flavonoids and polyphenols, with anti-inflammatory and antioxidant effects (Tissot et al., 2023). MS leaves are widely used in supplements, teas, and as a food source in regions where the tree grows. With approximately 28.2% protein content on a dry basis (Melesse et al., 2009), comparable to soybean meal, MS leaves help combat malnutrition, particularly among infants and nursing mothers (Derbo & Debelew, 2024). They offer higher vitamin C levels than oranges, higher levels of vitamins than carrots, greater calcium content than milk, more iron than spinach, and more potassium than bananas (Kumssa et al., 2017). Additionally, their protein quality is comparable to that of milk and eggs (Kumssa et al., 2017). Traditionally, boiled leaf extracts are used to alleviate high blood pressure, and studies have shown that aqueous extracts can lower blood glucose and cholesterol levels (Ghebreselassie et al., 2011). MS leaves also exhibit anti-hyperglycemic and anti-hyperlipidemic effects in animal models, indicating potential in diabetes treatment (Toma et al., 2012). Furthermore, they serve as

livestock feed, especially during dry seasons, and are incorporated into skincare products for their anti-aging and moisturizing properties (Gonzalez et al., 2015).

- Seeds: MS seeds, easily identifiable due to their oblong or triangular shape and three papery wings, are rich in proteins, healthy fats, and bioactive compounds like glucosinolates and polyphenols (Seifu, 2015). These seeds contribute to cardiovascular health and may have anti-cancer properties (Tesfaye et al., 2022). MS seeds are also known for their natural coagulant properties, making them effective in water purification (Seifu, 2015). Studies show that MS seeds provide about 83% of the amino acids recommended for children aged 2 to 5, exceeding WHO/FAO standards (Adugna, 2016). MS seed oil, constituting about 45% of the seed, is high in oleic acid (76%) and exhibits excellent stability against oxidative rancidity, making it ideal for use in food, cosmetics, and pharmaceuticals (Abiyu et al., 2018; Ntila, 2017). The oil-extracted meal, rich in protein, is also valuable as livestock feed and in biodiesel production. MS seeds have antimicrobial properties, effective against pathogens like *Shigella spp.*, *Salmonella typhi*, and *Candida albicans* (Walter et al., 2011), and show potential in water treatment, reducing turbidity and bacterial contamination by up to 99.9% (Mmelesi et al., 2021). Additionally, MS seed powder can remove cadmium ions from water, reducing concentrations by 53.8% (Mataka et al., 2014). Research has also shown that MS seed extracts exhibit hypotensive, antihyperglycemic, and antimicrobial properties, with traditional uses for treating stomach aches and aiding postpartum recovery (Seifu, 2015; Abiyu et al., 2018; Mmelesi et al., 2021). The nutritional profile of MS seeds highlights their potential as an alternative protein source, with protein quality comparable to traditional animal-based sources, supporting the increasing demand for sustainable and functional food ingredients (Siddiqui et al., 2024).

2.3 MS Seeds as Alternative Sources of Protein

Protein is an essential nutrient that is fundamental to life and found in nearly all living organisms (Corsetti et al., 2024). It helps to maintain cellular, tissue, organ, and overall bodily functions (Chen & Liu, 2016). Alongside carbohydrates and fats, proteins are vital macromolecules in the human diet, meeting metabolic requirements and aiding in the synthesis of hormones, enzymes, and tissues (Akanji & Frayn, 2024). Day et al. (2022) reported that proteins from plant and animal sources provide crucial nutrients while remaining free of harmful byproducts, making them invaluable in functional food applications. These proteins also contain bioactive peptides specific sequences within their structure that are initially inactive but, once released through digestion, offer significant physiological benefits (Aluko, 2015). These peptides have been associated with lowering the risk of cardiovascular diseases, inhibiting tumor development, and managing hyperglycemia (Aluko, 2012; Aluko, 2015).

Animal-based foods such as eggs, meat, and fish have long been the primary sources of dietary protein. However, the rapid increase in global population has raised concerns about meeting the growing demand for protein. As a result, there is a growing focus on identifying alternative protein sources, particularly from plants and microbes. Foods that derive more than 12% of their energy from protein are considered excellent sources of this nutrient (Hagos et al., 2018). Among plant-based options, MS seeds are notable for their high-quality amino acid composition, making them a suitable choice for human consumption and an effective alternative to traditional protein sources (Abiyu et al., 2018). Following oil extraction, the protein content of MS seed meal increases to approximately 55.6% (Ntila, 2017), underscoring its potential as a sustainable protein option, especially in areas with limited access to animal proteins. The seeds, which contain 30–35% protein by weight, are comparable to the more widely recognized *Moringa*

oleifera (Adugna et al., 2016). Adugna et al. (2016) further explained that MS seeds offer a balanced mix of essential and non-essential amino acids, making them a complete protein source. Additionally, their high digestibility makes them an excellent dietary choice for both children and adults, particularly in regions affected by malnutrition (Abiyu et al., 2018).

MS seeds are abundant in bioactive compounds that significantly boost their nutritional value (Dessalegn & Rupasinghe, 2021). These include antioxidants, which play a vital role in mitigating oxidative stress and supporting overall health (Tefera, 2023). Furthermore, the seeds contain relatively low levels of anti-nutritional factors such as phytates, which enhance mineral bioavailability and further improves their nutritional profile (Raimi & Arire, 2024). The proteins and bioactive compounds in MS seeds are linked to numerous health benefits, such as antimicrobial and antioxidant activities, enhanced immune function, and support for tissue repair (Seifu, 2015). Furthermore, studies indicate that certain compounds in MS seeds may help regulate blood sugar levels, highlighting their potential as a dietary aid for managing diabetes (Shumi, 2019). With their high-quality protein content, MS seeds may serve as an effective remedy for addressing protein-energy malnutrition (PEM), especially in developing regions. Their rich amino acid composition supports growth, immune health, and tissue repair, while their superior bioavailability ensures optimal nutrient absorption (Nathaniel, 2021). As a sustainable and easily accessible protein source, MS seeds offer significant promise in combating global malnutrition.

2.4 Protein Isolation

Isolating proteins from MS seeds can be achieved using various methods, each with unique benefits that influence the quality and type of proteins obtained. The choice of extraction technique is critical, as it significantly impacts both the protein yield and the resulting functional and structural properties. Commonly used methods for extracting proteins include the following:

2.4.1 Alkaline extraction coupled with isoelectric pH precipitation (ISO)

Alkaline extraction is a widely used method for isolating proteins, involving the adjustment of the protein solution's pH to an alkaline range, typically between 9 and 11, using a solution such as sodium hydroxide (NaOH) (Hewage et al., 2022). This high-pH environment facilitates dissociation of proteins from the surrounding matrix, enabling extraction of soluble proteins. The mixture is subsequently processed through centrifugation or filtration to remove insoluble components, such as fibers, lipids, and other non-protein substances, resulting in a protein-enriched supernatant (Sultan et al., 2024). Once the proteins are solubilized in the alkaline medium, the solution's pH is reduced to the isoelectric point (pI), which is the pH at which the protein has no net charge and becomes insoluble (Yasothai & Giriprasad, 2015). This is achieved by gradually lowering the pH using an acid like hydrochloric acid (HCl) until it reaches the pI, typically around 4.5 to 6.0 for most plant proteins. At this stage, proteins precipitate out of the solution as their solubility diminishes. The precipitated proteins are collected by centrifugation, effectively separating them from the remaining soluble components. Although alkaline extraction is effective for dissolving and extracting proteins, it has some limitations. High-pH conditions can lead to alkaline hydrolysis, causing uncontrolled peptide bond cleavage and protein denaturation (Shi & McHugh, 2023). Additionally, the process may result in the co-precipitation of non-protein components, reducing the purity of the final protein product (Pessoa et al., 2024). Despite these drawbacks, it remains a widely employed technique for its efficiency in isolating proteins (Miranda et al., 2022).

2.4.2 Salt precipitation (SP)

This approach effectively isolates proteins from complex mixtures, such as plant-based sources like moringa seeds, by enabling targeted extraction and concentration under controlled

conditions. SP also known as salting out utilizes the addition of salts, such as NaCl, to a protein solution to selectively precipitate proteins (Gimenes et al., 2021). The introduction of NaCl raises the ionic strength of the solution, disrupting protein hydration shells and causing aggregation and precipitation (Ding et al., 2024). The mechanism involves dissolved ions from the salt competing with proteins for water molecules, reducing protein solubility and leading to their precipitation (Acosta & Sundar, 2019). Once the salt is added, the mixture is centrifuged to separate the precipitated proteins from the remaining soluble components. The resulting precipitate contains proteins selectively removed due to their diminished solubility in the high-ionic-strength environment.

2.4.3 Micellar precipitation (MP)

MP is a protein isolation method based on the solubility characteristics of proteins in salt solutions. This technique is particularly effective for isolating proteins that are highly soluble in concentrated salt solutions but become insoluble when the salt concentration is reduced (Wong et al., 2018). The process begins by dispersing protein flour in a concentrated salt solution, facilitating protein dissolution through a mechanism known as "salting-in (Ismail & Smith, 2024)." Once the proteins are solubilized, the solution is diluted with distilled water to decrease the salt concentration. This reduction in ionic strength triggers the formation of micelle-like aggregates as the protein molecules cluster together. These aggregates are then easily separated from the solution via centrifugation, making the process efficient for protein recovery. The precipitated proteins can subsequently be re-dispersed in an aqueous solution with a pH and ionic strength optimized for high solubility. If necessary, residual acids, bases, or salts used during the precipitation can be eliminated through dialysis or filtration, ensuring the purity of the isolated protein. This approach allows for the selective precipitation of proteins, leading to high purity, as it targets proteins with

specific solubility characteristics (Wong et al., 2018). This makes it suitable for isolating proteins without significant contamination from other components.

2.4.4 Membrane ultrafiltration (MU)

Membrane ultrafiltration (MU) is a separation technique that uses a semi-permeable membrane to isolate proteins based on their molecular weight. In this process, a protein solution is forced through the membrane, which has pores that selectively retain larger proteins while allowing smaller molecules, such as proteins and salts, to pass through. For example, when using a membrane with a 5 kDa cutoff, proteins smaller than 5 kDa will pass through the membrane, while those larger than 5 kDa will be retained. MU is especially effective for concentrating proteins or separating smaller compounds from larger proteins (Ratnaningsih et al., 2021). The larger proteins are retained by the membrane, while the smaller molecules pass through, resulting in a concentrated protein solution. This solution can either be used directly or undergo further processing for various applications.

Different methods of protein isolation yield varying protein contents in plant-based isolates. For example, chickpea, faba bean, lentil, and pea protein isolates obtained via ISO have protein contents ranging from 82-88%, which is typically higher than the 73-82% protein content achieved through salt extraction (Karaca & Nickerson, 2011). Similarly, soy protein isolates produced by ISO contain between 82-86% protein (Branch & Maria, 2017), while *Moringa oleifera* protein isolates obtained using the same method have been reported to reach up to 93.98% protein content (Aderinola et al., 2020). In an alternative technique, Boye et al. (2010) isolated lentil seed proteins using ultrafiltration, filtering a solubilized protein solution at pH 6.0 through a 50 kDa molecular weight cutoff membrane. This resulted in protein contents of 82.7% for red

lentils and 88.6% for green lentils. Alonso-Miravalles et al. (2019) employed a 10 kDa membrane for ultrafiltration, obtaining an even higher protein content of 93.7% in the lentil protein isolate.

2.5 Defatting Method

Before isolating proteins, certain preliminary techniques are often employed that can significantly influence the protein's structure and functionality. One key method is defatting, which involves removing lipids and other non-protein substances from food materials, particularly those of plant origin, prior to protein isolation (Fatima et al., 2024). This process is usually carried out with organic solvents like hexane, acetone, or ethanol. The main goal of defatting is to enhance the purity and functionality of the protein by eliminating fats and oils that may interfere with the protein extraction process or negatively impact the final product's performance in food applications (Mahfoud et al., 2023). However, the defatting process can also affect protein functionality by altering characteristics such as solubility, emulsifying properties, and thermal stability (Yue et al., 2021). Depending on the solvent used, lipid removal can lead to structural changes, including the increased exposure of hydrophilic or hydrophobic groups (Lam et al., 2018). These alterations affect how the protein interacts with water, fats, and other molecules (Yue et al., 2021). As a result, defatting can either improve or impair the protein's functional properties, such as its ability to stabilize emulsions, form gels, or interact with other components in food formulations.

Fang et al. (2023) found that protein isolates extracted from undefatted hemp seeds using reverse micelles (nanoparticles composed of organic solvents) had higher gelling concentration and stronger gel strength compared to those from defatted seeds. On the other hand, Yue et al. (2021) demonstrated that defatted oat protein isolates had a higher protein content (around 87%) than those from undefatted oats. Jeong et al. (2021) explored the effects of various solvents ethanol, hexane, and acetone on the functionality of protein isolates from edible insects. Their study

revealed that ethanol was the most efficient solvent for lipid removal, yielding the highest protein concentrations. These findings underscore how different solvents can affect protein structure and functionality in unique ways. The solvent's polarity, strength, and ability to solvate or remove lipids play a role in how it interacts with proteins (Corradi et al., 2019). For instance, non-polar solvents like hexane primarily target lipids and have minimal impact on protein structure, whereas polar solvents like ethanol may cause some degree of protein denaturation, altering solubility and functional properties (Kumoro et al., 2022). While removing lipids can improve protein purity and make it more suitable for uses such as emulsions, gels, and foams, harsh defatting conditions or improper solvents may damage the protein's structure and diminish its functional potential (Yue et al., 2021). Therefore, selecting the right solvent and optimizing defatting conditions are essential to achieving the desired protein characteristics for specific applications.

2.6 Structural-Functional Properties of Food Proteins

Proteins are essential not only for supplying a variety of amino acids in the human diet but also for their critical roles in food formulations due to their functional properties, which are significantly influenced by their structural characteristics (Nasrabadi et al., 2021). Protein structure is intricate and organized into four hierarchical levels: primary, secondary, tertiary, and quaternary (Koch & Schäfer, 2018). The primary structure represents the linear arrangement of amino acids joined by peptide bonds, which plays a critical role in defining the protein's function by influencing its folding into higher-level structures (Khan et al., 2017). The 20 amino acids that make up this sequence each have distinct side chains (R groups) that affect the protein's chemical properties, including solubility, charge, and reactivity (Khan et al., 2017). The secondary structure pertains to localized regions of the protein chain that form specific shapes, such as α-helices, β-sheets, and turns, due to hydrogen bonding between the backbone atoms (Smetana & Misra, 2017). These

structures are crucial for the protein's functional properties, such as its ability to form gels or emulsions. The tertiary structure describes the complete three-dimensional configuration of a single protein molecule, shaped by interactions among the side chains of its amino acids (Rehman et al., 2022). These interactions include hydrophobic interactions, hydrogen bonds, disulfide bonds, ionic bonds, and van der Waals forces (Rehman et al., 2022). The folding of the protein into its tertiary structure is essential for its biological activity, such as enzyme function or receptor binding (Selvaraj et al., 2022). Proteins from plant sources differ in structure due to variations in their polypeptide sequences and native environments (Day et al., 2022). These differences result in varying proportions of secondary and tertiary structures, ultimately affecting their functional properties. The dynamic conformation of a protein determines its functionality, including its digestibility, ability to be fragmented into peptides, and the availability of essential amino acids (EAAs) for nutrition (Day et al., 2022).

The term "protein functionality" can be somewhat uncertain, as its definition may vary depending on the context (Foegeding & Davis, 2011). However, it generally refers to how proteins behave as colloidal biopolymers, either independently or in combination with other ingredients in a food system (Foegeding & Davis, 2011). This behavior is influenced by the protein's intrinsic and extrinsic physicochemical properties, rather than its biological functions or nutritional roles (Zhang et al., 2021). Key functional properties of proteins include water and fat binding, viscosity, solubility, emulsification, foam formation, film formation and gelation. These functionalities are influenced by several molecular properties of the proteins, including hydrophobicity, surface charge, molecular weight, isoelectric point, surface topology, as well as their secondary and tertiary structures (Asen & Aluko, 2022). The specific state of the protein whether native, intermediate, or denatured can further influence these properties (Akharume et al., 2021).

Environmental factors such as pH, temperature, and ionic strength can cause structural alterations in proteins, impacting their functionality, the quality of food products, their nutritional value, and gastrointestinal availability (Aluko, 2024). Understanding the structure-function relationship of proteins is essential for analyzing their behavior in food systems. This involves not only examining the native structural conformations of proteins but also assessing how these structures evolve during denaturation and under various processing conditions. Moreover, the functional properties of food proteins are heavily impacted by the method employed for protein extraction as shown in Table 2.1. The following sections discuss the functional properties of proteins in more detail.

2.6.1 Solubility properties

Solubility refers to the ability of a protein to dissolve in a solvent, which is fundamental to other functional properties like emulsification, gelation, and foaming. Solubility is mainly determined by the protein's amino acid composition and molecular structure (Hossain et al., 2017). pH plays a significant role, as proteins typically exhibit low solubility at their isoelectric point, which is the pH at which the protein has a net charge of zero (Asen & Aluko, 2022). At certain pH levels, proteins can denature and aggregate, leading to reduced solubility. Ladjal-Ettoumi et al. (2016) found that lentil protein isolates show maximum solubility away from their isoelectric point, particularly in acidic or alkaline conditions, which enhances emulsification and gelation. Soybean protein isolates have peak solubility around pH 4-5, with solubility decreasing near their isoelectric point at pH 4.5 (Verfaillie et al., 2023). Similarly, pea proteins exhibit the highest solubility at pH 3 and pH 9, with a reduction in solubility at neutral pH, and insolubility at pH 4.5, which is likely due to aggregation at the isoelectric point (Lan et al., 2018).

2.6.2 Gelling properties

Gelation is the process by which proteins form a gel, occurring when protein molecules unfold and interact, creating a network that traps water (Asen et al., 2023). This process is vital for developing textures in various food products, influencing water and oil retention, texture, mouthfeel, and product stability (Akharume, et al., 2021). Gelation is especially important for foods that require structural integrity, such as tofu, puddings, and plant-based meats (Akharume, et al., 2021). Key factors influencing gelation include protein concentration, pH, ionic strength, and heat, while structural properties like protein size and its ability to unfold also play a role (Tanger etal., 2022). For instance, chickpea proteins form stronger gels at higher concentrations and weakly acidic or alkaline pH, where protein unfolding and cross-linking are maximized (Patil et al., 2024). Likewise, mung bean proteins show enhanced gelation at alkaline pH, where protein unfolding and the formation of gel networks are most efficient (Nie et al., 2022).

2.6.3 Emulsifying properties

Emulsifying properties describe a protein's capacity to facilitate the formation and stabilization of emulsions, which are mixtures of oil and water phases (McClements & Jafari, 2018). This functionality depends on factors such as protein structure, concentration, solubility, and processing techniques (Tang, 2017). Emulsifiers play a vital role in food products like dressings, whipped toppings, and mayonnaise, where they enhance texture and stability (Huamaní-Perales et al., 2024). Proteins function as emulsifiers due to their amphiphilic nature, possessing both hydrophilic (water-attracting) and hydrophobic (water-repelling) regions that allow interaction with both water and oil (Sani et al., 2024). To evaluate emulsifying properties, the Emulsion Activity Index (EAI) and Emulsion Stability Index (ESI) are commonly employed. The EAI quantifies a protein's ability to emulsify oil per unit of protein, while the ESI measures the emulsion's stability over time (Nasrabadi & Ghosh, 2024). The emulsifying characteristics of

legume proteins are shaped by both intrinsic factors, such as the plant source, and extrinsic factors, including processing conditions (Akharume et al., 2021). Emulsion stability is generally enhanced when disulfide bonds form within the proteins (Fan et al., 2022). In contrast, unstable emulsions may result from electrostatic repulsion, leading to intermolecular disulfide bonds (Nasrabadi & Ghosh, 2024). Emulsions are typically more stable at pH levels that are either acidic or alkaline, away from the isoelectric point, indicating the crucial role of pH in determining both emulsion capacity and stability (Chukwuejim & Aluko, 2024). Research indicates that pea protein isolates form the most stable emulsions at acidic pH levels, likely due to enhanced electrostatic repulsion, which prevents droplet coalescence (Burger & Zhang, 2019). Lentil protein isolates exhibit similar behavior, with optimal emulsion stability occurring at pH values away from their isoelectric point (Lee et al., 2021).

2.6.4 Foaming properties

Proteins can trap air and form stable foams, a property crucial in products like whipped cream, meringues, and beer (Laing et al., 2022). This foaming ability relies on the protein's capacity to quickly unfold and create interfacial layers that capture air. Key factors such as solubility, pH, and surface hydrophobicity play a critical role in this process (Laing et al., 2022). Foaming properties are typically assessed using two main indices: foam capacity (FC) and foam stability (FS). Foam capacity refers to the protein's ability to generate foam under specific conditions, including concentration and pH, and is expressed as the percentage increase in foam volume after homogenization. Foam stability, in contrast, measures the protein's ability to maintain the foam structure over a given time, typically ranging from 0 to 30 minutes. Proteins with strong foaming capacity and stability are especially suited for use in bakery products, meringues, and whipped toppings (Alavi et al., 2020). Foaming also influences texture in low-

density foods (Manbeck et al., 2017). Chickpea protein isolate exhibits a high foaming capacity at alkaline pH, where protein denaturation exposes hydrophobic regions that help stabilize air pockets (Wang et al., 2022). Similarly, mung bean protein isolate shows enhanced foam stability at elevated temperatures, where increased surface activity aids in maintaining foam structure over time (Yang et al., 2023).

2.6.5 Water holding capacity

Water-holding capacity (WHC) refers to a protein's ability to absorb and retain water within its structure, which directly affects the texture and juiciness of food products (Osemwota, 2021). A high WHC is especially important in products like meat analogs, where it helps maintain moisture, contributing to a desirable texture and mouthfeel (Osemwota, 2021). Additionally, WHC plays a role in viscosity and gelation in food formulations. Several factors influence WHC, including the protein’s surface area, the presence of hydrophilic groups, and environmental conditions like ionic strength and pH (Akharume et al., 2021). Proteins' ability to absorb and retain water depends on the presence of exposed polar groups in the protein’s polypeptide chain (Alabi, 2023). Tian et al. (2022) found that proteins with more hydrophobic surfaces have a lower WHC due to their reduced ability to trap water. Similarly, lentil proteins demonstrate greater WHC due to their structural flexibility, which aids in water retention and supports juiciness in plant-based meat applications (Webb et al., 2023).

2.6.6 Oil holding capacity

Oil Holding Capacity (OHC) refers to a protein's ability to absorb and retain oil within its structure, which plays a key role in flavor retention and texture enhancement in food products (Asen et al., 2023). A high OHC improves the flavor profile and mouthfeel, making the protein

ideal for high-fat products like mayonnaise and salad dressings (Wang, 2018). It also contributes to the texture and consistency of baked goods and desserts. The OHC is influenced by the hydrophobicity of the protein surface, which is determined by its amino acid composition and structural conformation (Malomo et al., 2014). OHC is largely driven by hydrophobic interactions that help retain oil in fat-rich food formulations (Batbayar, 2022). Protein isolates from flaxseed, rapeseed, sunflower, and soybean exhibit high OHC due to their hydrophobic amino acid content, which enhances oil retention and contributes to a smooth, rich mouthfeel in dairy alternatives (Ngo, 2023). Likewise, chickpea proteins are well-suited for salad dressings and emulsions, where their high OHC is crucial for flavor retention and stability (Meganaharshini, 2023).

Table 2.1 outlines the effects of different protein extraction methods on the functional properties of plant-based protein isolates, demonstrating significant differences influenced by both the extraction technique and the plant source. The findings highlight the critical role of extraction methods in determining protein functionality. Protein solubility shows considerable variation depending on the extraction process and source. For example, ISO provided greater solubility than SP in proteins from pea, faba bean, and chickpea (Karaca & Nickerson, 2011). In contrast, SP performed better than MP for pea proteins (Stone et al., 2015). In lentils, MU achieved higher solubility than ISO (Alonso-Miravalles et al., 2019). Regarding WHC, MP extraction yielded better results than ISO for bambara groundnut and pea proteins (Adebowale et al., 2011; Stone et al., 2015). However, ISO surpassed SP in lentils (Osemwota et al., 2021). For emulsifying properties, ISO consistently outperformed SP in pulse proteins (Karaca & Nickerson, 2011), reflecting its ability to maintain the protein’s hydrophilic-lipophilic balance, which is essential for emulsion stability. The least gelation concentration, which indicates a protein's gel-forming capability, was lower for MU than ISO in pulses and lentils (Boye et al., 2010; Joshi et al., 2011).

For *Moringa oleifera*, SP achieved a lower LGC compared to ISO (Aderinola et al., 2020), suggesting that the extraction process affects protein denaturation and cross-linking behavior, which in turn impacts gelation. OHC was higher for ISO than MP in bambara groundnut proteins (Adebowale et al., 2011). However, for lentils, MU extraction outperformed ISO, indicating that extraction methods influence protein-lipid interactions differently (Alonso-Miravalles et al., 2019). Lastly, foaming properties were superior in ISO-extracted proteins compared to those obtained via MP and SP for pea and lentil proteins (Stone et al., 2015; Osemwota et al., 2021). This could be attributed to ISO's ability to better preserve the protein structure and surface activity.

Table 2.1: Effects of protein extraction methods on functional properties of plant-based protein isolates

Functional properties	Source	Isolation techniques	Refeerence
Protein solubility	Pea proteins, faba bean, and chickpea	ISO > SP	Karaca & Nickerson (2011)
	Pea	SP > MP	Stone et al. (2015)
	Lentils	MU > ISO	Alonso-Miravalleset al. (2019)
Water holding capacity	Bambara groundnut	MP > ISO	Adebowale et al. (2011)
	Pea	MP > ISO	Stone et al. (2015)
	Lentils	ISO > SP	Osemwota et al. (2021)
Emulsifying properties	Pulse	ISO > SP	Karaca & Nickerson (2011)
Least gelation concentration	Pulse	MU < ISO	Boye et al. (2010)
	Lentil	MU < ISO	Joshi et al. (2011)
	Moringa oleifera	SP < ISO	Aderinola et al. (2020)
Oil holding capacity	Bambara groundnut	ISO > MP	Adebowale et al. (2011)

	Lentils	MU > ISO	Alonso-Miravalleset al. (2019)
Foaming properties	Pea	ISO > MP	Stone et al. (2015)
	Lentils	ISO > SP	Osemwota et al. (2021)

2.7 Bioactive Peptides

Bioactive peptides are usually embedded within the primary structure of food proteins and remain inactive until released through enzymatic hydrolysis (Aluko, 2015). Once released, these peptides can exhibit various biological activities, including inhibiting critical metabolic enzymes, lowering the risk of cardiovascular diseases, and preventing the proliferation of cancer cells, among other effects (Nwachukwu & Aluko, 2019). The release of bioactive peptides occurs through hydrolysis, a process that can be achieved through various methods:

- Chemical Hydrolysis: This method involves breaking down proteins using strong acids, bases, or chemical reagents like hydrochloric acid or sodium hydroxide to break peptide bonds (Aluko & Aluko, 2012). Although efficient and cost-effective, chemical hydrolysis can be harsh and non-specific, often leading to the degradation of some peptides and a loss of their functional properties (Aluko, 2012). The extreme conditions, including high temperatures or pH levels, may also cause unwanted side reactions, reducing the quality of the hydrolysate. Despite these drawbacks, chemical hydrolysis is often used in large-scale production where precision is less of a concern (Kadam et al., 2024).
- Microbial Fermentation: In this method, microorganisms such as bacteria, fungi, or yeasts decompose proteins into smaller peptides that offer health benefits and amino acids through enzymatic action. Microbial proteases catalyze the hydrolysis of proteins during fermentation, making it a more environmentally friendly alternative to chemical hydrolysis (Cruz-Casas et al., 2021). Microbial fermentation can be optimized to generate specific peptides with desired properties, but it requires careful control of factors like pH, temperature, and fermentation duration to ensure the desired level of hydrolysis (Cruz-Casas et al., 2021).

- Enzymatic hydrolysis: This is the most employed method for breaking down proteins into smaller fragments, primarily due to its precision, mild reaction conditions, and ability to generate bioactive peptides (Kadam et al., 2024). This process uses proteolytic enzymes (proteases) to cleave proteins into peptides that exhibit bioactivity, as these functional peptides are inactive within the intact protein structure (Aluko, 2015). Once released from the protein matrix, these peptides exhibit their physiological functions (Aluko, 2015). Mohanty et al. (2016) reported that certain proteins, such as casein in milk, contain amino acid sequences that become bioactive peptides when cleaved by specific enzymes. Proteases like alcalase, flavourzyme, pepsin, trypsin, and pancreatin are commonly used in protein hydrolysis, with each enzyme targeting specific peptide bonds, producing unique peptide profiles in the hydrolysate. Alcalase, a bacterial protease, has broad specificity for hydrophobic amino acids, making it effective for generating peptides with antioxidant and antihypertensive properties (Aluko, 2015). Flavourzyme, derived from fungi, exhibits both endopeptidase and exopeptidase activities, allowing it to release smaller peptides and free amino acids that enhance the hydrolysate's flavor profile (Azman et al., 2023). Pepsin, a gastric enzyme, preferentially cleaves peptide bonds involving aromatic amino acids like phenylalanine, tryptophan, and tyrosine, mimicking initial protein digestion in the stomach (Wang, 2022). Trypsin, a pancreatic enzyme, specifically cleaves peptide bonds at the carboxyl side of arginine and lysine, producing peptides with unique bioactivities (Wang, 2022). Pancreatin, a blend of digestive enzymes, includes proteases that further degrade proteins into peptides and amino acids, improving the digestibility and bioavailability of the hydrolysate (Ahmed et al., 2022). The choice of enzyme is critical in developing hydrolysates with targeted functional properties, as the enzyme's specificity determines the

type of peptides produced (Aluko, 2015). Enzymatic hydrolysis can be precisely controlled by manipulating parameters such as enzyme concentration, temperature, pH, and reaction time, enabling the production of peptides with specific bioactivities, including antioxidant, antihypertensive, and antimicrobial effects (Kadam et al., 2024).

2.8 Bioactive Properties of Protein Hydrolysates

As illustrated in Table 2.2, bioactive peptides can provide various physiological effects that support health. The health benefits associated with bioactive peptides include:

2.8.1 Antioxidant properties

Normal cellular metabolism generates reactive oxygen species (ROS) and reactive nitrogen species (RNS), highly reactive molecules that can cause significant damage to cellular components (Aluko, 2012). These reactive species can target and degrade essential cellular structures, including DNA, proteins, and lipids within cell membranes, contributing to the development and progression of chronic diseases such as cancer, kidney failure, atherosclerosis, Alzheimer's disease, and cardiovascular disorders (Nwachukwu & Aluko, 2019). To counteract the harmful effects of ROS and RNS, the body synthesizes a range of antioxidant enzymes and compounds that neutralize these reactive species, thereby protecting cells from oxidative stress and reducing the risk of oxidative damage-related diseases (Kadam et al., 2024). However, the body's antioxidant defense system can be compromised by factors such as aging, genetic predispositions, and environmental stressors (Aluko, 2012). This disruption can lead to an imbalance, with the overproduction of ROS and RNS surpassing the body's ability to neutralize them, thereby amplifying cellular damage (Mei, 2019). To restore balance, incorporating external (exogenous) antioxidants, such as antioxidant-rich peptides, is crucial. These exogenous antioxidants can complement the body's natural defenses, helping to mitigate oxidative stress and reduce the risk of diseases associated

with insufficient endogenous antioxidant activity (Aluko, 2015). This approach provides a potential strategy for managing and preventing the harmful effects of oxidative stress-related conditions. Some of the common methods used to measure the antioxidant activity of peptides include:

- ROS Scavenging: Peptides are assessed for their ability to scavenge or neutralize reactive oxygen species (ROS), which are highly reactive molecules that can damage cells and tissues (Mundi & Aluko, 2014). Common ROS measured include hydroxyl radicals (OH), hydrogen peroxide (H_2O_2), perhydroxyl or superoxide radicals (OOH), and DPPH (2,2-diphenyl-1-picrylhydrazyl).
- ORAC (Oxygen Radical Absorbance Capacity): The ORAC assay evaluates the total antioxidant capacity of a substance by determining its ability to neutralize oxygen radicals (Nwachukwu & Aluko, 2019). A higher ORAC score indicates greater antioxidant potential, and this test is frequently used to compare antioxidant effectiveness across different compounds.
- Metal Ion Chelation: Some peptides demonstrate antioxidant activity by binding with metal ions, which play a role in free radical formation through processes such as the Fenton reaction (Mundi & Aluko, 2014). By chelating these metal ions, peptides can help prevent harmful free radical generation.
- Inhibition of Lipid Peroxidation: Peptides are also tested for their capacity to inhibit lipid peroxidation, which involves free radical attacks on lipids and can cause cellular damage (Kadam et al., 2024). Linoleic acid, a polyunsaturated fatty acid, is commonly used as a model in these tests, and the ability to inhibit its oxidation is a strong indicator of antioxidant efficacy (Aluko, 2015).

The antioxidant potential of peptides is primarily influenced by their amino acid composition. Amino acids such as cysteine, methionine, histidine, and tryptophan, which contain sulfur, nitrogen, or functional groups, play a key role in enhancing their ability to scavenge free radicals and metal ions (Aluko, 2012). The arrangement of these amino acids within the peptide chain also influences its antioxidant capabilities. For example, cysteine has a thiol group (-SH) that can donate electrons to neutralize reactive species (Aluko, 2012). Methionine can act by donating electrons and breaking down peroxides (Alabi, 2023). Histidine, with its metal-binding capacity, further supports the peptide's role in preventing oxidative damage (Kadam et al., 2024).

2.8.2 Antihypertensive properties

Hypertension, or high blood pressure, is a significant health issue that heightens the risk of heart disease, stroke, kidney damage, and other severe conditions. Blood pressure regulation involves a complex network of processes, with the renin-angiotensin system (RAS) being one of the most crucial. This hormone system is central to managing blood pressure and fluid balance by influencing blood volume and the constriction of blood vessels, both of which directly impact blood pressure levels (Moussa et al., 2023). The RAS system is activated when blood pressure is perceived as low, initiating mechanisms to elevate it (Aluko, 2015). Key components of this system include proteins and enzymes such as angiotensinogen, renin, angiotensin I, angiotensin-converting enzyme (ACE), and angiotensin II. As shown in Figure 2.1, angiotensinogen, a precursor protein produced in the liver, is released into the bloodstream in an inactive form and does not directly influence blood pressure under normal conditions (Samsamikor, 2023). However, when blood pressure drops or blood flow to the kidneys decreases, the kidneys release an enzyme called renin into the bloodstream (Olarewaju et al., 2018). Renin acts on angiotensinogen, converting it into angiotensin I, an inactive precursor. Angiotensin I circulates in the blood without

directly affecting blood pressure until it is converted into its active form by Angiotensin-Converting Enzyme (ACE). ACE, primarily found in the lungs and other tissues, transforms angiotensin I into angiotensin II, the active molecule (Mundi & Aluko, 2014). Angiotensin II is a powerful vasoconstrictor, causing blood vessels to narrow, which increases vascular resistance and elevates blood pressure (Aluko, 2021). Additionally, angiotensin II stimulates the adrenal glands to release aldosterone, a hormone that prompts the kidneys to retain sodium and water, further increasing blood volume and contributing to higher blood pressure (Khalid et al., 2022). Antihypertensive peptides, found in natural sources such as milk, fish, and plants, help reduce blood pressure by targeting components of the renin-angiotensin system, particularly ACE (Aluko, 2015). These peptides inhibit activities of renin and ACE, preventing the production of angiotensin I and conversion to angiotensin II, respectively, which reduces vasoconstriction and lowers blood pressure. Prominent sources of antihypertensive protein hydrolysates include plants like rapeseed, hempseed, wheat bran, soy, sesame, and *Moringa oleifera* (He et al., 2013; Malomo et al., 2015; Daliri et al., 2019; Zou et al., 2020; Aondona et al., 2021; Bassogog et al., 2024).

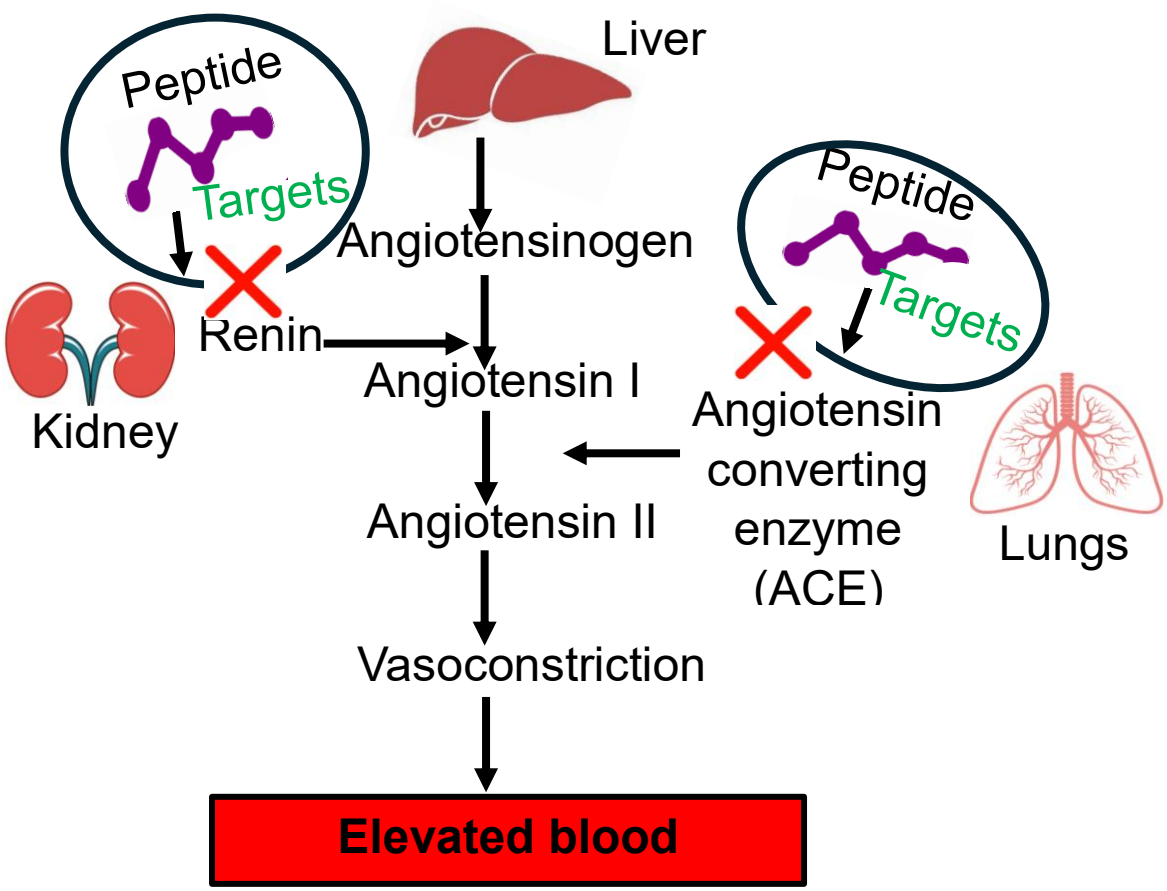

Figure 2. 1: Renin-Angiotensin System for blood pressure regulation

2.8.3 Anti-cancer properties

Protein hydrolysates may exhibit anti-cancer properties due to the bioactive peptides they contain, which can influence cellular mechanisms involved in cancer progression (Kadam et al., 2024). These peptides have demonstrated potential to suppress cancer cell proliferation, trigger apoptosis (programmed cell death), inhibit metastasis, and regulate immune system responses (Kadam et al., 2024). The anti-cancer effects are often linked to their antioxidant capabilities, ability to neutralize free radicals, and regulation of signaling pathways critical for cancer cell survival (Aluko, 2015). Certain peptides have been shown to interfere with cancer cell proliferation and reduce estrogen receptor activity, which is significant in specific types of cancer (Aluko, 2012). Additionally, peptides have demonstrated the ability to inhibit the growth of colorectal and breast cancer cell lines, likely through their antioxidant properties and immune-modulating effects (Yeo & Shahidi, 2021). For example, peptides derived from wheat germ protein hydrolysates have been found to reduce leukemia cell viability by causing cell cycle arrest and promoting apoptosis in cancer cells (Ortiz-Martinez et al., 2014).

2.8.4 Antidiabetic and anti-obesity properties

Peptides have shown significant effectiveness in inhibiting key metabolic enzymes, aiding in the management of chronic diseases such as obesity and diabetes (Antony & Vijayan, 2021). Diabetes mellitus, a metabolic disorder caused by insulin deficiency, is marked by persistent hyperglycemia, which disrupts carbohydrate, fat, and protein metabolism (Mukhtar et al., 2020). Globally, diabetes ranks as the fourth leading cause of mortality (Saeedi et al., 2020). Obesity, defined by Ellulu et al. (2017) as excessive fat accumulation posing serious health risks, results from a complex interplay of genetic, dietary, lifestyle, and environmental factors that create a prolonged energy imbalance (Franzago et al., 2020). These conditions are major public health

challenges, often interconnected and contributing to secondary health problems such as cardiovascular diseases. A promising therapeutic strategy for managing obesity and diabetes involves the inhibition of digestive enzymes such as α-amylase, α-glucosidase, and pancreatic lipase. These enzymes are critical for breaking down carbohydrates and fats, and their inhibition helps regulate blood sugar levels and reduce fat absorption (Sultana et al., 2020). Pharmaceutical options such as acarbose (an α-glucosidase inhibitor) and orlistat (a lipase inhibitor) have been developed for this purpose (Liu et al., 2023). However, these drugs are associated with side effects and may be cost-prohibitive, especially in developing countries (Blahova et al., 2021). Consequently, there is growing interest in natural alternatives, particularly bioactive peptides derived from plant proteins, as a safer and more accessible option. The natural structure of peptides makes them ideal for metabolism within the body, allowing them to be efficiently broken down and utilized without accumulating to toxic levels (Akbarian et al., 2022).

Unlike synthetic drugs, which often require specific metabolic pathways for clearance, peptides are processed in a way that minimizes the risk of tissue buildup and associated harm. Peptides have several advantages over conventional pharmaceuticals:

- Efficient absorption: The human digestive system contains specialized transporters that facilitate the rapid and effective absorption of peptides, transferring them directly into the bloodstream (Aluko, 2012). This efficient mechanism ensures peptides are quickly distributed throughout the body. In contrast, many drugs rely on passive diffusion or need to be lipophilic (fat-soluble) for proper absorption, often requiring more complex processes for uptake (Dima et al., 2024).
- Rapid metabolism and low toxicity: Once absorbed, peptides are readily hydrolyzed by physiological enzymes, leading to a short duration of action (Udenigwe & Aluko, 2012).

This rapid breakdown prevents peptides from lingering in the body, reducing the risk of tissue damage or adverse side effects. Conversely, pharmaceutical drugs often remain in the body for extended periods, increasing the potential for toxicity and organ damage, particularly with chronic use (Aluko, 2015).

- Maximal impact on kidneys: Peptides are broken down into by-products that are easily eliminated through normal metabolic processes, sparing the kidneys from strain (Li et al., 2024). Unlike many drugs that rely on renal excretion and can contribute to kidney damage, peptides are a safer alternative, particularly for individuals with existing kidney conditions (Aluko, 2019).

- Lower risk of side effects with higher doses: While peptides may not match the immediate potency of synthetic drugs, their lower risk of toxicity allows them to be administered in higher doses to achieve similar therapeutic effects (Kadam et al., 2024). This makes them a safer option for long-term use.

- Integration into foods: One of the most appealing aspects of peptides is their potential to be incorporated into everyday food products, such as beverages, cheeses, and baked goods (Kumar et al., 2024). This compatibility with regular diets provides a natural, convenient, and less invasive method of delivering health benefits compared to traditional pharmaceutical treatments.

The versatility of peptides, combined with their ability to target specific sites of action, makes them highly promising therapeutic agents. Their effectiveness is closely linked to size, sequence, and other structural characteristics (Tacias-Pascacio et al., 2020).

Table 2.2 highlights the bioactive properties of plant-based protein hydrolysates, providing a comprehensive overview of their biological activities, sources, enzymes used, and relevant studies. Plant proteins such as lupin, wheat gluten, and chickpea have demonstrated significant anti-aging and anti-inflammatory effects. The use of enzymes like flavourzyme, alcalase, pepsin, and trypsin enhances the release of peptides with these bioactivities. Antioxidant peptides have been isolated from a variety of plant sources, including canola meals, mushrooms, and *Moringa oleifera* seeds. Alcalase is the most frequently used enzyme, often in combination with others such as chymotrypsin and pancreatin, to optimize peptide release. Studies like those by Hunsakul et al. (2022) and Famuwagun et al. (2021) highlight the role of enzymatic specificity in targeting oxidative stress. Antihypertensive peptides are well-documented in legumes like kidney beans, hemp seeds, and bambara ground nuts; these peptides inhibit ACE activities. Enzymes such as alcalase and pepsin are pivotal in generating ACE-inhibitory peptides, as evidenced by Mundi & Aluko (2014) and Malomo et al. (2020). Protein hydrolysates derived from yellow field peas, sesame, and potato tubers show promising antidiabetic properties, often mediated by peptides that inhibit enzymes involved in glucose metabolism. Studies like those by Bassogog et al. (2024) and Awosika & Aluko (2019) highlight the diverse enzymatic treatments and their resultant bioactive profiles. Anti-obesity peptides have been identified in soy, black beans, and other legumes, targeting mechanisms such as lipase inhibition or appetite regulation. Enzymes like bromelain and actinase are used alongside alcalase to enhance peptide activity, as noted by Toledo et al. (2016) and Mirzapour-Kouhdasht et al. (2022). Anti-cancer peptides have been isolated from amaranth and soybean. Enzymatic hydrolysis using alcalase, pepsin, and trypsin facilitates the release of bioactive peptides with anti-proliferative effects on cancer cells, as shown by Ramkisson et al. (2020) and Das et al. (2022). Alcalase emerges as a predominant enzyme, indicating its broad

applicability and efficiency in hydrolyzing diverse plant proteins. Enzyme combinations further enhance activity by targeting specific peptide bonds. Legumes, seeds, and grains are frequently studied sources, offering a rich reservoir of bioactive peptides. Lesser-known sources like amaranth and watermelon seeds show untapped potential. Bioactivity often depends on the peptide sequence, which is influenced by the protein source and enzymatic treatment (Nwachukwu & Aluko, 2019). Tailoring these factors is crucial for targeted health benefits.

Table 2.2: Bioactive properties of plant-based protein hydrolysates

Biological activities	Sources	Enzymes used	References
Anti-aging and anti-inflammatory properties	Lupin	Izyme and alcalase	del Carmen Millán-Linares et al. (2014)
	Wheat gluten	Alcalase	Montserrat-de La Paz et al. (2020)
	Chickpea	Alcalase, pepsin, and trypsin	Serrano-Sandoval et al. (2023)
Antioxidant properties	Canola meal	Alcalase, chymotrypsin, pepsin, trypsin, and pancreatin	Alashi et al. (2014)
	Mushroom	Alcalase, pancreatin, and flavorzyme	Kimatu et al. (2017)
	Moringa oleifera seed	Alcalase	Aderinola et al. (2019)
	Mungbean	Alcalase, neutrase, protamex, and papain	Xie et al. (2019)
	Amaranth leaf	Pepsin, trypsin, alcalase, and chymotrypsin	Famuwagun et al. (2021)
	Jasmine rice bran	Alcalase and flavorzyme	Hunsakul et al. (2022)
Antihypertensive	Kidney bean	Alcalase	Mundi & Aluko (2014)

	Hemp seed	Pepsin, alacalse, papain, and pepsin + pancreatin	Malomo et al. (2015)
	Bambara	Alcalase, trypsin, and pepsin	Arise et al. (2017)
	Mungbean	Alcalase	Sonklin et al. (2018)
	Fluted pumpkin	Pepsin and alcalase	Malomo et al. (2020)
	Pigeon pea	Pepsin and pancreatin	Olagunju et al. (2022)
	Pea	Flavorzyme	Xia et al. (2022)
Antidiabetic	Yellow field pea	Chymotrypsin, pepsin, trypsin, and alcalase	Awosika and Aluko (2019)
	Potato tubers	Alcalase	Marthandam Asokan et al. (2019)
	Sesame	Pepsin + pancreatin	Idowu et al. (2021)
	Moringa oleifera seed	Alcalase, trypsin, thermolysin, and pepsin	Bassogog, et al. (2024)
Anti-obesity	Soy	Alcalase	Lammi et al. (2015)
	Black bean	Alcalase and bromelain	Toledo et al. (2016)
	Chickpea, lentil, fava bean, and green pea	Alcalase, pepsin + pancreatin	Moreno et al. (2020)
	Amaranth	Chymotrypsin, bromelain, and actinase	Ajayi et al. (2021)

	Watermelon seed	Alcalase, proteinase, and actinidin	Mirzapour-Kouhdasht et al. (2022)
	Oat bran	Alcalase	Esfandi (2023)
Anti-cancer	Amaranth	Alcalase, pepsin, and trypsin	Ramkisson et al. (2020)
	Soybean	Alcalase	Das et al. (2022)

2.9 Bitter Taste in MS

Although MS seeds offer significant health and nutritional benefits, utilization is often hindered by their natural bitterness. This bitterness can deter consumers and complicate the incorporation of MS into food products, especially for those seeking pleasant-tasting protein sources. The bitter flavor is attributed to a combination of bioactive compounds, including alkaloids, saponins, glucosinolates, and polyphenols, each contributing uniquely to the overall taste profile and medicinal properties of MS (Wang et al., 2022). To reduce the bitter taste in MS seeds, various processing methods can be employed, including soaking and leaching, boiling, roasting, and fermentation. Effectively reducing the bitterness of MS seeds requires a strategic combination of these methods, aimed at targeting the specific compounds responsible for their bitter taste. When used together, these approaches can significantly improve the flavor, making the seeds more appealing and versatile for both culinary and nutritional applications.

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CHAPTER THREE

MANUSCRIPT ONE

3.0 EFFECTS OF SOLVENT TYPE ON THE STRUCTURAL AND FUNCTIONAL PROPERTIES OF PROTEIN ISOLATES OBTAINED FROM DEFATTED *MORINGA STENOPETALA* SEED MEAL

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3.1 Introduction

The rising popularity of plant-based proteins is driven by increased consumer awareness of their health benefits and the urgent need for sustainable food sources. Plant proteins serve as an excellent alternative to animal proteins, offering both nutritional value and unique functional characteristics (Sha & Xiong, 2020). These functional properties such as emulsifying and foaming properties are critical for determining their suitability for food and nutraceutical applications (Galanakis, 2021). However, the effectiveness of these proteins largely depends on their structural integrity, which is often altered during processing (Nunes & Tavares, 2019). Proteins generally remain in their native state, but processing steps during isolation can denature them, resulting in a loss of functionality. One significant step is defatting, the removal of lipids, which plays a pivotal role in altering protein structure and performance. Various organic solvents, such as acetone, ethanol, and hexane, are often adopted to remove fat components. Hexane, a frequently utilized solvent in the food industry, presents concerns owing to residual traces, which may pose health risks (Cravotto et al., 2022). Ethanol, on the other hand, is a safer alternative and has been successfully applied to defat soybeans, maize, and *Quercus suber* fruits (Kwiatkowski & Cheryan, 2002; Ferreira-Dias et al., 2003; L'hocine et al., 2006). Ethanol and isopropanol have also been used to defat yellow mealworm protein, while hexane was found to improve functional properties in insect proteins (Mishyna et al., 2019). In response to concerns about residual solvents, cold-pressure extraction has emerged as an alternative method to reduce oil content and minimize protein denaturation, as demonstrated in studies on *Hermetia illucens* larvae (Kim et al., 2022). Defatting is particularly crucial for oil-rich seeds, such as *Moringa stenopetala* (MS), which contain approximately 45% crude oil (Fang et al., 2023). This chapter explores how different defatting methods affects the functional properties of MS proteins.

MS is a drought-resistant, long-living deciduous tree native to Ethiopia and Kenya, also known as the African Moringa or cabbage tree (Leone et al., 2015). A member of the monogeneric Moringaceae family, the tree is known for its bottle-shaped trunk, twisted seed pods, and cabbage-like edible leaves, which give it the name "cabbage tree." (Mikore & Muluget, 2017; Boopathi & Abubakar, 2021; Rezaei et al., 2022). It can live for over 100 years, continuously producing leaves and seeds that are dispersed by wind or water (Boopathi & Abubakar, 2021). Beyond its resilience to water stress, MS serves as an important protein source, playing a crucial role in enhancing food security and improving nutrition (Horn et al., 2022). The seeds of MS contain approximately 36% protein and include all essential amino acids, making them a promising source of functional protein isolate (Melesse et al., 2011; Demise et al., 2021). With a balanced amino acid composition, MS protein isolate (MSPI) offers potential applications in human foods, providing an alternative to other plant or animal-based protein sources. The protein composition of MS seeds was compared to the FAO's reference, hen's egg protein, revealing higher total amino acid content but lower essential amino acids (Olaofe et al., 2013). These findings highlight MS seeds' potential for food applications, especially in underutilized plant-based food systems. The proteins derived from MS seeds are also highly digestible, making them an accessible nutritional source (Seifu, 2015). This combination of digestibility and a complete amino acid profile enhances the value of MS in addressing protein deficiencies and combating malnutrition. Due to their nutrient-rich composition, MS seeds can also be used for animal feed, further extending their impact on food security (Stadtlander and Becker, 2017).

In the food industry, protein ingredients are valued for their structure, essential functionalities, and nutritional benefits. Functional properties are directly linked to a protein's secondary structure and surface hydrophobicity (Nwachukwu & Aluko, 2021). Previous research

reports have indicated that the nutritional value and functionality of proteins are influenced by their structural composition (Aryee et al., 2018; Wang et al., 2022; Asen et al., 2023). Reported studies have extensively explored the structure-function relationship in various plant proteins. For example, *Moringa oleifera*, a closely related species has been well-researched for its protein functionality and potential application in food formulations (Mune et al., 2016; Jain et al., 2019; Aderinola et al., 2020; Bassogog et al., 2022; Fatima et al., 2023). Research has shown that the seed proteins of *Moringa oleifera* exhibit excellent water and oil absorption capacities, which are critical for developing meat analogs, baked goods, and emulsified products like sausages and mayonnaise (Aderinola et al., 2020; Barbhai et al. 2024). Additionally, Moringa oleifera protein isolates demonstrate significant solubility over a broad pH range (Illingworth et al., 2024), making them suitable for incorporation into acidic beverages, protein shakes, and other liquid food systems. Studies also highlight its remarkable foaming and emulsifying properties, attributed to its ability to stabilize oil-water interfaces and produce stable foams (Aderinola et al., 2020; Illingworth et al., 2024), which are essential for creating whipped toppings, ice creams, and certain baked products. Furthermore, enzymatic hydrolysates of Moringa oleifera proteins have shown improved digestibility and enhanced bioactivity, such as antioxidant and antihypertensive properties, making them ideal for use in nutraceuticals and functional foods (Ma et al., 2021; Kumar et al., 2022). These findings align with the general functional properties observed in other food proteins, such as their role in texture modification, moisture retention, and enhancing the sensory qualities of various food products. However, similar research on MS remains limited. Despite its potential as a sustainable plant-based protein with bioactive and nutritional properties, the functional performance of MSPI has not been sufficiently explored, especially concerning the effect of defatting methods on their structure and functionality.

In addition to the functional properties of proteins, sensory quality is a critical attribute, particularly when considering the five basic flavors that define human taste: bitterness, sourness, sweetness, saltiness, and umami (Ma et al., 2024). Traditionally, human sensory panels comprising both trained and untrained panelists are employed to assess the taste of food products (Jiang et al., 2018). However, conducting and training sensory panels can be time-consuming and costly. Furthermore, if panelists are not adequately trained, their evaluations may introduce biases. As a result, many researchers have turned to the electronic tongue (e-tongue) as a rapid, unbiased, and cost-effective alternative to human sensory panels (Schlossareck & Ross, 2019). Bitterness is affected by several factors, such as the presence of specific compounds like polyphenols, alkaloids, and glucosinolates (Liu et al., 2024). In plant-based proteins, such as those derived from Moringa, bitter compounds tend to be concentrated in the protein isolate during extraction (Wong et al., 2023). The defatting process plays a crucial role in determining the bitterness of protein isolates. The method used for defatting can either concentrate or reduce these bitter-tasting compounds, thereby impacting the overall sensory quality of the protein isolate. This consideration is especially relevant in product development, where the level of bitterness can significantly influence consumer acceptance. By carefully selecting appropriate defatting methods, it is possible to optimize the sensory profile of protein isolates, achieving a balance between functional properties and desirable taste characteristics.

Based on our understanding, this is the first study to analyze the structural and functional properties of *Moringa stenopetala* seed protein isolates and to investigate the impact of various defatting solvents on these properties. While numerous studies have focused on the leaves of MS, limited attention has been given to the protein isolates derived from its seeds. Given the variability in defatting techniques and the absence of comparative studies on MS protein isolates, it is crucial

to examine how different defatting methods influence their functional properties. The current gap in research hinders our understanding of how processing steps, particularly defatting, affect the structural integrity and functionality of MS proteins. This study seeks to address this gap by offering a comprehensive evaluation of how different defatting methods impact the functional properties of MSPI. The findings will provide valuable insights for optimizing defatting protocols to preserve and enhance the performance of MS proteins as suitable ingredients for food product formulations. Additionally, this work contributes to the development of sustainable plant-based protein alternatives, aligning with the growing demand for environmentally friendly and nutritionally beneficial food products.

3.2 Materials and Methods

3.2.1 Raw materials and sample preparation (defatting process)

MS seed meal that was produced through mechanical oil press was obtained from BioTEI Inc. (Winnipeg, MB, Canada) and stored at -20°C. Double-distilled water (DDW) produced using a Millipore Milli-Q™ water purification system (Millipore Corp., Milford, MA, USA) was utilized throughout the research. Methanol, acetone, hexane, anhydrous ethanol and all analytical grade chemicals used were obtained from Fisher Scientific (Oakville, ON, Canada) or Sigma Aldrich (St. Louis, MO, USA). The MS meal was milled using a coffee grinder (PG-13658FA-CAN) to obtain a fine flour. As shown in Figure 3.1, the milled flour was defatted at room temperature using seven different solvents: methanol, acetone, hexane, anhydrous (100%) ethanol, 50% ethanol, 70% ethanol, and water. A flour-to-solvent ratio of 1:10 (w/v) was maintained throughout the process. Each mixture was prepared in triplicate, stirred for 1 h, and then allowed to settle. The solvent phase was carefully decanted into storage containers, and the extraction process was repeated for an additional 30 min. The solvent from the second extraction was combined with the initial extract.

The defatted flour was spread thinly on an aluminum plate and left to dry overnight at room temperature in a fume hood. The dried flour was weighed and stored in an airtight container at -20°C, along with the undefatted meal for comparison. The solvent from the pooled extract was evaporated using a rotary evaporator under vacuum at 40°C and 100 rpm. The resulting residue (oil) was collected and analyzed for fatty acid profile.

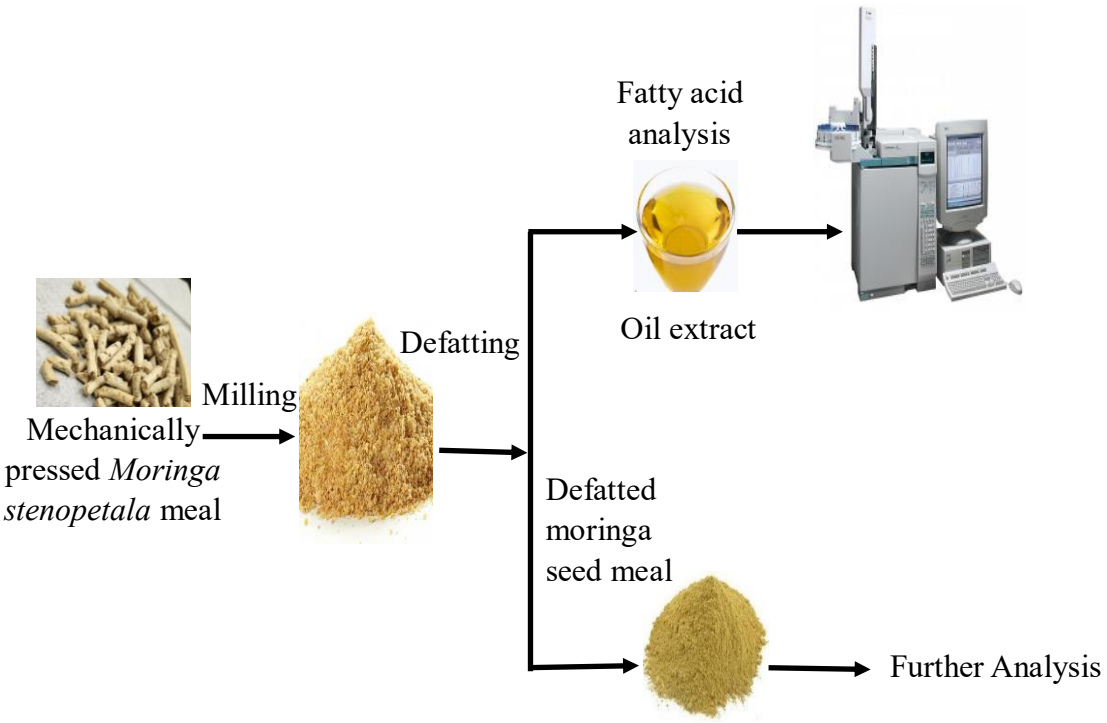


Figure 3. 1: Defatting process of MS seed meal

3.2.2 Lipid extraction for fatty acid analysis

Lipid was extracted following the method described by Garcés and Mancha (1993). A total of 100 mg of the oil samples were placed into three sets of 12 ml tubes. To each tube, 5 ml of chloroform-methanol (2:1) and 1 ml of 0.025% calcium chloride were added. The sample tubes were vortexed for 20 seconds and centrifuged at 2000 rpm for 12 min. The samples were then stored overnight in a refrigerator. The bottom phase was transferred to new, clean 12 ml tubes and dried under nitrogen in a 40°C water bath

3.2.3 Lipid methylation and gas chromatography

Lipid methylation and gas chromatography were done as described by Bartle and Myers (2002). The heating block was preheated to 110°C. A volume of 1.5 ml of Boron trifluoride (BF_3) (14% w/w) and 2 ml of hexane were added into 12 ml tubes containing the dehydrated liquid oil. The tubes were wrapped with Teflon tape to ensure a tight seal and were capped tightly. They were then placed in the heating block for 1 h and 15 min, followed by cooling for 10 min. After cooling, 1 ml of double-distilled water (DDW) was added to the tubes, which were capped and vortexed for 20 s. The mixture was centrifuged at 2000 rpm for 12 min. The upper phase was carefully transferred to a pre-weighed set of 12 ml tubes. The samples in these tubes were dried under nitrogen in a 40°C water bath until only the lipid remained. The tubes containing the lipids were weighed, and the lipid weight was calculated. Hexane was then added to each tube to achieve a concentration of 10 mg/mL of the sample. Finally, 1.5 ml of the 10 mg/mL sample was transferred into a GC (Gas Chromatography) vial, and each vial was analyzed using a GC-FID (Gas Chromatography-Flame Ionization Detector). The methylated samples were analyzed using a DB225MS column (30 m x 0.25 mm diameter, 0.25 mm film thickness; Agilent Technologies Canada Inc., Mississauga, Ontario) on a Varian 450 GC equipped with a Flame Ionization Detector

(FID). The temperature program began at 70°C for 1 min, which was then raised to 180°C at 25°C/min and held for 1 min, followed by an increase to 200°C at 10°C/min and held for 1 min. The temperature was subsequently increased to 220°C at a rate of 2°C/min and maintained for 6 min. It was then raised to 240°C at 20°C/min and held for 20 min. The total analysis time was 46.40 min, with a 20:1 split ratio and a column flow rate of 1.3 ml/min. Hydrogen served as the carrier gas. Fatty acids were identified by comparing their retention times to those of known standards and quantified based on the peak area using calibration curves prepared from reference fatty acid methyl esters.

3.2.4 Protein isolation using alkaline solubilization followed by pH isoelectric precipitation

MS seed meal or defatted flour was dispersed in 1 M NaOH at a 1:5 (w/v) ratio and stirred continuously for 1 h (Figure 3.2). The mixture was later centrifuged at 8,000 × g for 1 h at room temperature to separate the supernatant. The supernatant was adjusted to pH 4.5 using 2.5 M HCl and refrigerated for 12 h to facilitate protein precipitation. Following refrigeration, the acidified mixture was centrifuged again at 8,000 × g for 1 h at 4°C. The resulting precipitate was freeze-dried to obtain *Moringa stenopetala* protein isolate (MSPI) and stored at -20°C. Protein yield was calculated by dividing the amount of protein in the MSPI after isolation (in grams) by the amount of protein in the moringa seed meal (in grams), and then multiplying the result by 100 to express it as a percentage. The MSPIs were labelled as follows based on the solvent used to defat the original meal: methanol (MMGPI), acetone (AMGPI), hexane (HMGPI), anhydrous ethanol (EMGPI), 50% ethanol (5EMGPI), 70% ethanol (7EMGPI), and water (WMGPI).

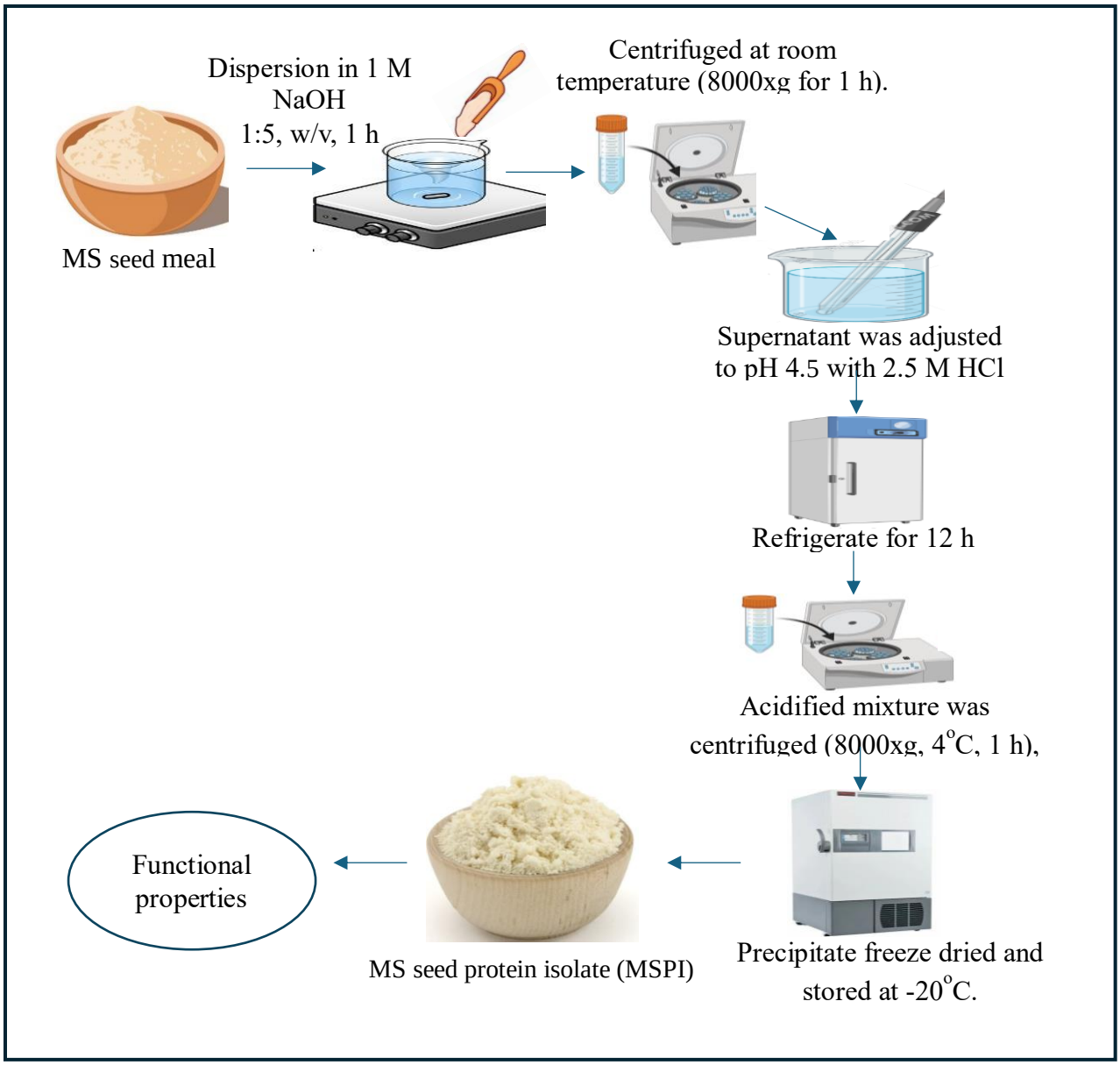


Figure 3. 2: Isolation of *Moringa stenopetala* (MS) seed proteins by alkaline solubilization followed by isoelectric pH precipitation.

3.2.5 Protein isolation using NaCl solubilization coupled with membrane isolation

MS seed meal or defatted flour was initially dispersed at a concentration of 5% (w/v) in a 0.3 M NaCl solution and stirred for 1 h to ensure thorough solubilization of the proteins. The mixture was then centrifuged to separate the solid residues from the supernatant. The supernatant, which contained the soluble protein fraction, was collected and subjected to ultrafiltration using a 5 kDa molecular weight cut-off (MWCO) membrane. During the diafiltration process, water was periodically added to the system. The filtration continued until the permeate appeared clear. The resulting precipitate was freeze-dried to obtain *Moringa stenopetala* protein isolate ($MSPI_{NaCl}$) and stored at -20°C (Figure 3.3).

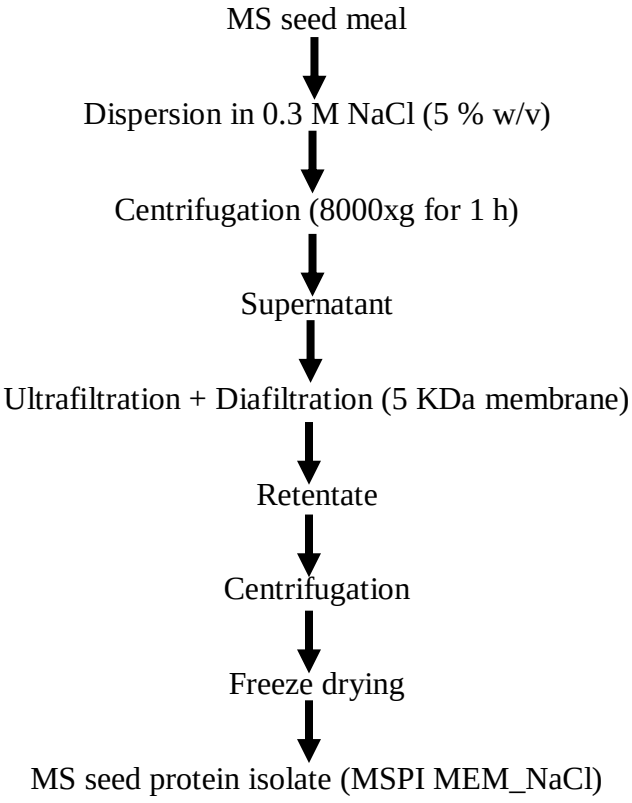


Figure 3. 3: Isolation of *Moringa stenopetala* (MS) seed protein by NaCl solubilization and membrane ultrafiltration

3.2.6 Proximate composition

The moisture, crude protein, dry matter, and ash contents of the protein isolate were determined using the methods specified by the Association of Official Analytical Chemists (Horwitz and Latimer, 2005). The crude fiber and fat contents were measured according to the procedures set by the American Oil Chemists' Society (Mehlenbacher et al., 2010).

3.2.7 Amino acid composition

The amino acid profile of the MSPIs was analyzed using the HPLC Pico-Tag system, following the method outlined by Bidlingmeyer et al. (1984). The samples were hydrolyzed with 6 M HCl for 24 h. Methionine and cysteine contents were determined after oxidation with performic acid, as described by Gehrke et al. (1985). The tryptophan content was measured after alkaline hydrolysis, following the procedure of Landry and Delhaye (1992).

3.2.8 Total polyphenol content

The total phenolic content of the protein isolates was quantified using the Folin-Ciocalteu method, following the procedure of Hoff and Singleton (1977) with slight modifications. A standard calibration curve was created using gallic acid concentrations ranging from 25 to 350 μg/mL in 50% (v/v) methanol. The protein isolates were diluted to concentrations between 600 and 1400 μg/mL in 50% methanol A 0.25 mL aliquot of either the gallic acid standard or protein isolates was combined with 0.25 mL of Folin-Ciocalteu reagent and incubated in the dark at room temperature for 5 min. Then, 0.5 mL of a 20% (w/v) sodium carbonate solution and 4 mL of distilled deionized water (DDW) were added to the mixture. After mixing thorough, the solution was incubated for 1 h in the dark. The absorbance of the resulting green color was measured at 725 nm using an Ultrospec UV-visible spectrophotometer (GE Healthcare, Montreal, PQ, Canada).

The total phenolic content of MSPI was expressed as milligrams of gallic acid equivalents (mg GAE) per gram of protein.

3.2.9 E-tongue testing

The sample was weighed and dispersed in double-distilled water at a concentration of 50 mg/g. It was then thoroughly mixed at room temperature for approximately 30 min to ensure complete extraction of the bitter compounds. After mixing, the solution was centrifuged at 5,000 x g to separate any insoluble particles. The clear supernatant was collected and filtered through 0.45 µm filter discs. The samples were equilibrated to room temperature prior to analysis. For the calibration and validation of the e-tongue, bitter standard solutions were prepared in reverse osmosis water at specific concentrations to serve as reference points for known bitterness levels. The standards included caffeine at 0.05 mg/mL, acetaminophen at 0.5 mg/mL, famotidine at 0.02 mg/mL and 0.05 mg/mL, quinine at 0.01 mg/mL and 0.04 mg/mL, loperamide hydrochloride at 0.005 mg/mL, and denatonium benzoate at 0.5 mg/mL. These standards provided consistent benchmarks for quantifying bitterness in test samples using a linear regression plot.

3.2.10 Intrinsic fluorescence

As outlined by Agboola and Aluko (2009), the intrinsic fluorescence of the isolated MS protein samples was measured using a JASCO FP-6300 spectrofluorometer (JASCO Corporation, Tokyo, Japan). A 10 mg sample of the protein was suspended in 1 mL of 0.1 M sodium phosphate buffer. The mixture was then centrifuged, and the supernatant collected and its protein content quantified. The supernatant was diluted to concentrations of 0.02% and 0.002% (w/v) using the same buffer (pH 3-8). The protein samples were excited at wavelengths of 257 nm (phenylalanine), 275 nm (tyrosine), or 295 nm (tryptophan), and emission intensity was measured up to 500 nm at 25°C. Fluorescence from the buffer blanks was subtracted from the corresponding sample

emissions. Fluorescence intensity (FI) was reported in arbitrary units, with the maximum FI during the wavelength scan noted as Fmax, and the corresponding wavelength as λmax.

3.2.11 Surface hydrophobicity

The surface hydrophobicity of MSPI was evaluated following the method described by Haskard and Li-Chan (1998), using 1-anilino-8-naphthalenesulfonate (ANS) as the fluorescent probe. A 10 mM phosphate buffer (pH 7.0) was prepared, and 10 mg of the protein isolate was dissolved in the buffer. The solutions were stirred at room temperature for 1 hour, then centrifuged at 7000 x g for 20 min. The supernatant was collected and diluted with 10 mM phosphate buffer (pH 7.0) to achieve protein concentrations between 30 and 250 µg/mL. A 40 µL aliquot of 8 mM ANS was mixed with the phosphate buffer and added to each 4.0 mL sample of protein solution. The excitation wavelength was set to 390 nm and emission wavelength to 470 nm using a spectrofluorometer (JASCO FP-6300). The fluorescence intensity (FI) versus protein concentration (mg/mL) was used to determine the protein surface hydrophobicity (So) through linear regression analysis.

3.2.12 Circular dichroism

Far- and near-UV circular dichroism (CD) spectra were obtained following the method outlined by Agboola and Aluko (2009), with slight modifications. A J-815 spectropolarimeter (JASCO Corporation, Tokyo, Japan) was used at 25°C. The far-UV CD spectrum was measured at a protein concentration of 2 mg/mL in 0.01 M phosphate buffer (pH 3.0–9.0) over a wavelength range of 190–240 nm, using a quartz cell with a 0.5 mm path length. The near-UV spectrum was recorded at a protein concentration of 4 mg/mL in 0.1 M phosphate buffer (pH 3.0–9.0) over the range of 250–320 nm, with a 1 mm path length quartz cell. CD spectra were averaged over three consecutive scans, with the corresponding buffer spectra automatically subtracted. Since the

samples contained protein mixtures, the resulting spectra reflected the combined conformational contributions of the different polypeptide components. The secondary structure of the protein fractions was analyzed from the far-UV data using the SELCON3 algorithm (optimized for 190–240 nm) for secondary structure prediction, as described by Lobley et al. (2002) and Whitmore and Wallace (2004), available through the DichroWeb platform. (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml).

3.2.13 Differential scanning calorimetry

Differential scanning calorimetry (DSC) analysis was performed following the method outlined by Freire (1995). Distilled water (10 mg) was placed into an aluminum pan, and 1 mg of the protein sample was added. The pan was then hermetically sealed. The sealed pans were heated at a rate of 10 °C/min, from 30 to 120 °C. An empty, sealed pan served as a reference. The denaturation temperature (T_a) and enthalpy change (ΔH) were determined using Universal Analysis 2000 software (Version 4.5). All experiments were performed in triplicate. Before thermal analysis, the sealed pans containing the protein isolate samples and buffers were equilibrated at 25°C for 2 hours.

3.2.14 Protein solubility

MSPIs solubility was assessed following the method previously described by Malomo et al. (2014), with slight modifications. 10 mg of each sample was dissolved in 5 mL of 0.1 M acetate buffer (pH 3 and 5), phosphate buffer (pH 7), or Tris buffer (pH 9) to achieve a 1% (w/v) protein concentration. The mixtures were thoroughly vortexed, allowed to hydrate for 1 hour, and then centrifuged at 5600 × g for 30 min. The protein concentration in the supernatants was measured using the modified Lowry method (Markwell et al., 1978). Similarly, the total protein content of the samples was determined by dissolving them in 0.1 M NaOH and analyzing them with the same

method. Absorbance readings at 660 nm were taken using a UV spectrophotometer to calculate the total protein content and solubility as follows:

$$Protein\ solubility\ (\%) = \frac{\text{Sample protein content in the supernatant}}{\text{Sample total protein content}} \times 100$$

3.2.15 Heat coagulability

Heat coagulability of MSPI was determined following the method outlined by Malomo et al. (2014). The protein isolates were dispersed in buffer at pH 3, 5, 7, and 9. A portion of each protein sample was then heated for 15 min in boiling water, followed by cooling to room temperature (23 – 25°C). After cooling, the samples were centrifuged at 5600 x g for 30 min. The supernatant was carefully collected, and its protein content determined using the Lowry method.

The heat coagulability calculation was done using this formula:

$$Heat\ coagulability\ (\%) = \frac{protein\ content\ of\ S1 - protein\ content\ of\ S2}{protein\ content\ of\ S1} \times 100$$

Where S1 represents the total protein content of sample (initial), and S2 represents the protein content of supernatant after heating (final).

3.2.16 Emulsion formation and stability

Oil-in-water emulsions were prepared with slight modifications to the method outlined by Chao et al. (2018). Protein slurries with concentrations of 10, 15, and 20 mg protein/mL were prepared separately in 0.1 M phosphate, acetate, or Tris buffer (pH 3.0, 5.0, 7.0, and 9.0). To create the emulsions, 1 mL of pure canola oil was mixed with 5 mL of protein slurry. The mixture was homogenized 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 average oil droplet size ($d_{3,2}$) was measured using a Mastersizer 3000 (Malvern Instruments Ltd., Malvern, UK), with Milli-Q water

as the dispersing medium. Each emulsion sample was continuously sheared and mixed with approximately 100 mL of distilled water using the small-volume wet dispersion unit (Hydro 3000) connected to the instrument until the desired obscuration level was reached. The oil droplet size of each emulsion was measured automatically in triplicate, and the average value was recorded. To assess the emulsion's stability, the $d_{3,2}$ was measured again 30 minutes after preparation. The emulsion stability was calculated using the following equation:

$$Emulsion\ stability\ (\%) = \frac{Oil\ droplet\ size\ at\ 0\ \text{min}\ \ (d3,2)}{Oil\ droplet\ size\ after\ 30\ \text{min}\ \ (d3,2)} \times 100$$

3.2.17 Foam formation and stability

Foam formation was carried out following the method described by Chao et al. (2018). Protein dispersions at concentrations of 10, 15, or 20 mg protein/mL were prepared by adding the samples into 50 ml graduated centrifuge tubes containing 0.1 M phosphate, acetate, or Tris buffer solutions at pH 3.0, 5.0, 7.0, or 9.0. The mixtures were homogenized at 20,000 rpm for 1 min using a 20 mm shaft on a Polytron PT 3100 homogenizer (Kinematica AG, Lucerne, Switzerland). The foam capacity, defined as the ability of the continuous phase to incorporate air, was determined as the mean of three measurements using the following equation:

$$\text{Foam Capacity}\ = \frac{\text{After whipping volume} - \text{Before whipping volume}}{\text{Before whipping volume}} \times 100$$

Foam stability was evaluated after 30 min at room temperature by measuring the volume of the remaining foam. This value was expressed as a percentage of the initial foam volume to assess the foam's capacity to retain its structure over time.

3.2.18 Water and oil holding capacity

The method described by Malomo et al. (2014) was used with slight modifications to determine the water and oil holding capacities of the protein isolate. Protein samples at concentrations of 20, 40, and 60 mg/mL based on protein content were dispersed into pre-weighed centrifuge tubes containing 0.1 M phosphate, acetate, or Tris buffer solutions at pH 3.0, 5.0, 7.0, or 9.0 for water holding capacity assessment. For oil holding capacity, protein samples at the same concentrations were dispersed in 10 mL of pure canola oil. The water and oil dispersions were vortexed for 1 minute and allowed to stand for 30 min before being centrifuged at 5600 × g for 30 min at room temperature. After centrifugation, the supernatant was carefully collected, and excess water or oil from the upper phase was drained for 15 min. The amount of water or oil retained per gram of sample was determined by weighing the centrifuge tubes containing the protein residue.

3.2.19 In vitro protein digestibility (IVPD)

A modified version of the method outlined by Hsu et al. (1977) was used to assess the IVPD. The enzyme system consisted of trypsin, chymotrypsin, and peptidase at concentrations of 1.6 mg/mL, 3.1 mg/mL, and 1.3 mg/mL respectively. A sample suspension was prepared by dissolving 10 ml of protein in double-distilled water (DDW) to achieve a concentration of 6.25 mg/mL. While stirring at 37°C, the suspension was adjusted to pH 8.0 using NaOH. The enzyme solution was kept chilled in an ice bath, and 1 ml added to the protein suspension. The pH change was monitored for 10 min, and the percentage of protein digestibility was calculated using the regression equation proposed by Hsu et al. (1977).

% Protein digestibility = 210.46 - 18.10Xf

where Xf represents the final pH value of each sample after digestion for 10 min.

3.2.20 Statistical analysis

Assays were conducted in triplicate to determine mean values and standard deviations. Statistical data analysis was performed using one-way and two-way analysis of variance (ANOVA). Significant differences between mean values were assessed at a 5% significance level using Duncan's multiple range tests. All statistical analyses were carried out using the IBM SPSS statistical package (version 24, Armonk, NY, USA) and GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA).

3.3 Results and Discussion

3.3.1 Fatty acid composition MS

The fatty acid composition of MS seed oil is summarized in Table 3.1. Fat was extracted from MS seed meal using different solvents including, acetone (AMG), ethanol (EMG), hexane (HMG), methanol (MMG), 50 % ethanol (5EMG), 70 % ethanol (7EMG), water (WMG), and the fatty acid composition of each lipid extract was compared with the fatty acid profile of the undefatted flour (UMG). It was observed that the fat extracted from WMG, 7EMG, 5EMG, and MMG did not yield sufficient or complete fatty acid profiles for reliable qualification. This may be due to the low efficiency of these polar solvents in extracting lipids, the extraction of non-lipid components, or degradation of fatty acids during the extraction process. The results show differences in saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and the n-6/n-3 ratio across the various defatted samples.

The total SFA content ranges from 24.54% - 25.06%. Notably, HMG exhibits the highest SFA content. Among the detected SFAs, stearic acid (C18:0) is the most abundant, ranging from 9.22% in UMG to 9.51% in AMG. This high level of stearic acid, which is known to have a neutral impact on cholesterol levels (Berry et al., 2009), suggests that MS seed could be valuable for functional food applications. The SFA content observed in this study is lower than reported values for dried fish (Sun, 2024) but slightly higher than those found in field peas (Solis, 2013). Notably, in contrast to this study, palmitic acid (C16:0) was reported as the most abundant SFA in dried fish (Sun, 2024). MUFA constituted the majority of the fatty acid content in all samples, with oleic acid (C18:1) being the most predominant, ranging from 71.771% in AMG to 72.242% in UMG. The slight reduction in oleic acid levels in the defatted samples suggests that the defatting process has minimal impact on this MUFA (Hammad et al., 2016). High MUFA levels, particularly oleic

acid, are linked to cardiovascular health benefits, including reduced LDL cholesterol and anti-inflammatory effects (Hammad et al., 2016; Lordan et al., 2017). Like PUFAs, MUFAs can lower unhealthy cholesterol levels and reduce the risk of heart disease. Both MUFAs and PUFAs are found in foods rich in vitamin E, a critical antioxidant often lacking in many diets (Mariamenatu & Abdu, 2021). However, MUFAs offer an advantage over PUFAs as they are more resistant to oxidation, making them more stable for storage and cooking (Lawrence, 2013; Asif, 2015; Shaheen et al., 2023). The MUFA content observed in this study is comparable to that reported for *Moringa oleifera* seed oil (Aly et al., 2016) and the seeds of *Moringa drouhardii* and *Moringa hildebrandtii* (Stadtlander & Becker, 2017).

PUFA levels were relatively low across the samples, ranging from 0.50% (AMG) to 0.55% (UMG). Linoleic acid (C18:2, n-6) was the dominant PUFA, while alpha-linolenic acid (C18:3, n-3) was detected in trace amounts (0.039–0.048%). HMG showed a slightly higher alpha-linolenic acid content (0.05%), marginally enhancing the n-3 PUFA profile. However, the overall low PUFA content suggests that MS seed oil is not a significant source of essential PUFAs. The PUFA levels reported here are significantly lower ($p < 0.05$) than those found in *Moringa oleifera* seed oil (9.44–10.44%) (Aly et al., 2016). The n-6/n-3 ratio is an important nutritional indicator, with lower ratios being associated with a reduced risk of cardiovascular diseases and metabolic disorders (Gebauer et al., 2019). In this study, the ratio decreased from 12.75 in UMG to 9.2 in HMG. This result revealed that defatting reduced the n-6/n-3 ratio. The favorable n-6/n-3 ratio in HMG suggests that hexane defatting slightly improves the nutritional profile of the meal, making it more suitable for functional food applications. The n-6/n-3 ratio observed in this study is comparable to that reported for soybean (Ivanov, et al., 2010). The overall fatty acid profile shows that non-polar solvents such as acetone, and hexane have minimal impact on the relative proportions of major

fatty acids. However, subtle differences, particularly in PUFA levels and the n-6/n-3 ratio, suggest that hexane extraction slightly enhances the nutritional value of MS seed oil. The high MUFA content retained across all samples further supports the potential of MS seed oil as a valuable ingredient in the formulation of functional foods.

Table 3.1: Fatty acid composition of oil extracts from undefatted MS meal (UMG) or meal defatted with; acetone (AMG), 100% ethanol (EMG), and hexane (HMG).

Fatty acids		UMG (%)	AMG (%)	EMG (%)	HMG (%)
Capric acid (SFA)	C10:0	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Lauric acid (SFA)	C12:0	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Myristic acid (SFA)	C14:0	0.15 ± 0.03	0.14 ± 0.00	0.14 ± 0.00	0.14 ± 0.03
Pentadecyclic acid (SFA)	C15:0	0.01 ± 0.02	0.01 ± 0.00	0.01 ± 0.00	0.011 ± 0.00
Palmitic acid (SFA)	C16:0	7.04 ± 0.00	6.73 ± 0.01	6.80 ± 0.00	6.75 ± 0.04
Margaric acid (SFA)	C17:0	0.09 ± 0.04	0.09 ± 0.01	0.09 ± 0.02	0.09 ± 0.02
Stearic acid (SFA)	C18:0	9.22 ± 0.00	9.51 ± 0.01	9.45 ± 0.00	9.49 ± 0.09
Arachidic acid (SFA)	C20:0	3.75 ± 0.00	4.00 ± 0.01	3.94 ± 0.00	3.98 ± 0.02
Behenic acid (SFA)	C22:0	3.12 ± 0.01	3.36 ± 0.02	3.30 ± 0.06	3.34 ± 0.00
Lignoceric acid (SFA)	C24:0	1.13 ± 0.01	1.22 ± 0.01	1.19 ± 0.09	1.21 ± 0.01

Total SFA		24.54 ± 0.02	25.09 ± 0.00	24.95 ± 0.05	25.05 ± 0.07
Trans-palmitoleic acid (MUFA)	C16:1t	0.28 ± 0.00	0.25 ± 0.00	0.25 ± 0.05	0.25 ± 0.00
Palmitoleic (MUFA)	C16:1	0.31 ± 0.00	0.29 ± 0.01	0.29 ± 0.06	0.29 ± 0.02
Oleic acid (MUFA)	C18:1	72.24 ± 0.02	71.77 ± 0.01	71.89 ± 0.03	71.78 ± 0.05
Vaccenic acid (MUFA)	C18:1n7c	0.60 ± 0.09	0.57 ± 0.05	0.57 ± 0.01	0.57 ± 0.02
Gondoic acid (MUFA)	C20:1	1.43 ± 0.08	1.49 ± 0.05	1.49 ± 0.00	1.50 ± 0.00
Erucic (MUFA)	C22:1	0.03 ± 0.01	0.03 ± 0.03	0.03 ± 0.03	0.03 ± 0.02
Total MUFA		74.90 ± 0.08	74.41 ± 0.05	74.53 ± 0.06	74.43 ± 0.00
Linoleic acid (PUFA)	C18:2	0.51 ± 0.03	0.45 ± 0.08	0.46 ± 0.08	0.46 ± 0.04
Alpha linoleic acid (PUFA)	C18:3n3	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.00
Total PUFA		0.55 ± 0.02	0.49 ± 0.07	0.50 ± 0.07	0.51 ± 0.02
Total n-6		0.51	0.46	0.47	0.46

Total n-3	0.04	0.04	0.04	0.05
Ratio n-6/n-3	12.75	11.50	11.75	9.2

Saturated fatty acid (SFA), Monounsaturated fatty acid (MUFA), Polyunsaturated fatty acid (PUFA), n-6 (omega-6 fatty acid), n-3 (omega-3 fatty acid). The data represent the mean values of duplicate readings with their corresponding standard deviations.

3.3.2 Protein content and yield of MSPI

The protein contents of the ISO and MEM_NaCl MSPI are summarized in Table 3.2. Across all samples, the ISO method consistently yielded higher protein content than the MEM_NaCl method. This suggests that isoelectric precipitation is more effective for concentrating MSPI. The superior performance of the ISO method may be attributed to its reliance on the intrinsic properties of proteins, particularly their solubility and charge behavior at specific pH levels, to achieve precipitation. In contrast, the MEM_NaCl method relies on protein solubility in the presence of salts, governed by the salting-in effect, which can only dissolve proteins that are soluble in salts. Additionally, the use of a 5 kDa molecular weight cutoff (MWCO) membrane in the MEM_NaCl method may influence protein recovery. This cutoff selectively retains proteins larger than 5 kDa while allowing smaller proteins to pass through, potentially leading to the loss of low-molecular-weight proteins during filtration. These factors collectively contribute to the lower protein content observed with the MEM_NaCl method compared to isoelectric precipitation. For instance, AMGPI prepared via the ISO method had a protein content of 83.45%, whereas the MEM_NaCl method yielded 72.83%. A similar trend was observed across the other isolates. In the ISO method, the highest protein contents were recorded for AMGPI, MMGPI, and HMGPI, with no significant ($p < 0.05$) differences between them. For the MEM_NaCl method, AMGPI exhibited the highest protein content. WMGPI had the lowest protein content (69.59%) under the ISO method, suggesting that non-polar solvents such as acetone and hexane may be more effective in removing lipids, enhancing protein extraction and purity of the isolated protein. UMGPI, which was not defatted, showed a lower protein content compared to some defatted isolates, highlighting the importance of defatting in improving protein isolation by reducing lipid interference. Interestingly, EMGPI exhibited similar protein content to UMGPI, while WMGPI and 5EMGPI showed the

lowest values across both methods. The low protein content of WMGPI in both methods suggests that some proteins may have been lost during water-based defatting. A comparison of ethanol concentrations revealed that 70% ethanol (7EMGPI) yielded higher protein content than both 50% ethanol (5EMGPI) and 100% ethanol (EMGPI), indicating that 70% ethanol improves defatting efficiency and protein recovery. This could be because a 70% ethanol concentration strikes a balance between polarity and non-polarity, making it effective at solubilizing both hydrophilic and lipophilic compounds.

As detailed in Table 3.2, the ISO method consistently achieved higher protein yields across all samples compared to MEM_NaCl, reinforcing the superior efficiency of the ISO method in minimizing protein losses during extraction. The ISO method leverages the solubility behavior of proteins at their isoelectric point, enabling effective precipitation with minimal interference from other components. Additionally, the absence of membranes or salt gradients helps minimize protein losses, making ISO a reliable technique for high protein yield. In contrast, the MEM_NaCl method quantifies proteins based on molecular weight (5 kDa), potentially missing smaller proteins that are not retained by the membrane. This limitation may explain the lower yields obtained using MEM_NaCl. The highest protein yield in both methods was observed for UMGPI. The absence of defatting in UMGPI likely preserved protein integrity, allowing higher recovery. In contrast, protein-lipid interactions and partial protein denaturation during defatting in other samples may have reduced extraction efficiency. Among the defatted samples, HMGPI and AMGPI achieved the highest yields under ISO, while 7EMGPI showed the highest value under MEM_NaCl. The lowest yield under ISO was recorded for 5EMGPI, suggesting that solvent concentration is critical to extraction efficiency. EMGPI, WMGPI, and MMGPI had the lowest values under MEM_NaCl, with no significant differences ($p < 0.05$) among them, indicating that

certain solvents might alter protein structure or remove protein components, depending on the method used.

Interestingly, Osemwota et al. (2021) reported a higher protein content for MEM_NaCl than ISO in lentil protein isolates, contrasting with the present study's findings. The ISO protein content observed here is slightly lower than that reported for pea protein isolates (Stone et al., 2015) and those of chickpea, lentil, pea, and soybean (Karaca et al., 2011). However, the ISO yields align closely with those for pea protein isolates. In contrast, the MEM_NaCl yields in this study were lower. For instance, an ISO-extracted amaranth protein concentrate yielded only 50.9% protein content and 19.1% yield at a pilot scale (Castel et al., 2012). These present results highlight the importance of defatting agents in eliminating lipid impurities, which enhances protein recovery. High protein content is crucial in food formulations as it improves nutritional value and functional properties such as solubility, emulsification, and foaming capacity (Ma et al., 2022). Protein isolates with high content are especially effective in products targeting muscle repair, weight management, and overall health improvement (Putra et al., 2021). The ISO method, which demonstrated superior efficiency in maximizing protein yield, is particularly suitable for studies where high protein recovery is critical. Consequently, all subsequent analyses in this study were performed using protein isolates obtained through the ISO method to ensure consistency and reliability in evaluating their structural and functional properties. This approach ensures not only optimal protein content but also enhances the quality and potential applications of the protein isolates in both food and nutraceutical contexts.

Table 3.2: Protein content (%) and yield (%) of protein isolates obtained from undefatted MS meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI).

Sample	Protein content (%)		Protein yield (%)	
	ISO (%)	MEM_{NaCl}	ISO	MEM_{NaCl}
UMGPI	74.01 ± 0.97^{cd}	44.82 ± 1.28^{f}	83.23 ± 3.32^{a}	63.67 ± 1.82^{a}
HMGPI	79.24 ± 4.02^{ab}	64.20 ± 1.26^{c}	68.53 ± 3.28^{b}	26.47 ± 0.52^{d}
7EMGPI	78.28 ± 1.53^{bc}	62.50 ± 0.20^{cd}	52.53 ± 4.56^{d}	37.54 ± 0.12^{b}
5EMGPI	69.5 ± 1.49^{d}	67.56 ± 1.55^{b}	41.73 ± 3.73^{e}	21.73 ± 0.50^{e}
EMGPI	73.92 ± 3.66^{cd}	58.45 ± 2.12^{e}	52.53 ± 3.32^{d}	12.92 ± 0.47^{f}
AMGPI	83.45 ± 2.74^{a}	72.83 ± 1.04^{a}	62.88 ± 1.95^{bc}	35.48 ± 0.51^{c}
WMGPI	69.59 ± 2.84^{d}	28.21 ± 3.40^{g}	53.04 ± 4.80^{d}	12.96 ± 1.56^{f}
MMGPI	80.24 ± 1.56^{ab}	59.99 ± 1.34^{de}	58.11 ± 4.42^{cd}	14.53 ± 0.33^{f}

ISO (Alkaline isoelectric pH precipitation), MEM_{NaCl} (NaCl membrane isolation). The data represent the mean values of duplicate readings with their corresponding standard deviations. Different superscripts within the same column signify statistically significant differences among the samples ($p < 0.05$). Protein content is reported on 'as is' basis while protein yield is based on the amount of protein in the defatted meal.

3.3.3 Proximate composition

Table 3.3 presents the proximate composition of MSPI obtained using various defatting methods. The composition highlights the differences in moisture, crude protein, fat, fiber, ash, and total solids content among the isolates. WMGPI exhibited the highest moisture content (3.34%), likely due to the use of water during the defatting process. Barla (2020) stated that water-defatting may expose hydrophilic regions within the protein matrix, enhancing water retention through increased protein-water interactions. This suggests that structural changes induced by water may promote moisture absorption. In contrast, 7EMGPI had the lowest moisture content, attributed to the combined effect of water and 70% ethanol, which efficiently extracted both lipids and polar components without excessive structural disruption. A lower moisture content improves the stability and shelf life of protein isolates, reducing microbial activity and spoilage risk (Joshi et al., 2011). The moisture content in this study aligns with the recommended range of 3 – 4 % for dried food products (Illingworth et al., 2024), demonstrating that MSPI yield dry protein isolates suitable for storage.

The crude protein content ranged from 84.36% to 96.95%. MMGPI had the highest protein concentration, suggesting that methanol defatting effectively retains proteins. The protein content was significantly higher ($p < 0.05$) from other isolates, including AMGPI and HMGPI, though these also exhibited high values. 5EMGPI displayed the lowest protein content among the isolates. The protein content of MS seeds is significantly higher compared to that of Moringa leaves, which range from 27.30% to 30.20% (Mikore & Mulugeta, 2017), highlighting the seeds as a richer source of protein. The protein content reported here is comparable to flaxseed protein isolates (90.6%) (Kaushik et al., 2016) and lentil protein isolates (Joshi et al., 2011). The fat content in MSPI ranged from 0.51% to 3.81%. Methanol and hexane defatting were particularly effective in

fat removal, leading to lower fat concentrations, which correlate with higher protein content. However, WMGPI retained the highest fat content, indicating that water defatting is less efficient for lipid extraction. Among the ethanol-treated isolates, 7EMGPI had the lowest fat content, suggesting that 70% ethanol provides the ideal polarity for efficient lipid removal and protein retention. The fat levels observed are higher than those reported for pea (Cui et al., 2020) and lentil protein isolates (Osemwota et al., 2021). These findings underscore the importance of solvent selection in tailoring the fat content of protein isolates for specific applications, such as low-fat formulations.

AMGPI and MMGPI yielded the highest fiber content, suggesting that both acetone and methanol extractions may have enhanced fiber-protein interactions. Acetone, being less polar, likely did not extract the fibers as efficiently as other polar solvents (Okolie et al., 2019). In contrast, 5EMGPI exhibited the lowest fiber concentration, which suggests that increased polarity of 50% ethanol may have facilitated more effective fiber removal during the defatting process. The fiber content of MSPI is similar to lentil protein isolates (Osemwota et al., 2021), suggesting that these isolates could provide added functionality in food applications. High fiber content enhances water-holding capacity, which is valuable for improving the texture of baked goods and meat alternatives (Hamad, 2021). Conversely, protein isolates with lower fiber content, such as 5EMGPI, are more suitable for beverages requiring better solubility and smoother textures (Boukid et al., 2021). Fiber also plays a critical role in digestive health by adding bulk to stool and preventing constipation (Ahmad & Al-Shabib, 2020).

The ash content in MSPI (4.21–9.90%) is superior to the 3.19–3.83% values that were reported for lentil protein isolates (Joshi et al., 2011) and other legumes such as soybeans, lentils, and chickpeas (Karaca et al., 2011). This highlights the mineral-rich composition of MSPI, which

can enhance its potential in nutraceutical applications targeting populations with specific mineral deficiencies (Gharibzahedi & Jafari, 2017). Minerals are essential for maintaining electrolyte balance, supporting enzymatic reactions, and promoting overall health (Thirulogasundar,, 2023). 5EMGPI had the highest ash content, indicating a higher concentration of minerals and inorganic compounds. In contrast, MMGPI exhibited the lowest ash concentration, indicating higher dissolution of minerals during the defatting process. The ash content in 5EMGPI and WMGPI was found to be higher than that of UMGPI, while the ash content in AMGPI, EMGPI, MMGPI, HMGPI, and 7EMGPI was lower than in UMGPI. This difference could be explained by the effect of non-polar solvents such as hexane and acetone used during defatting. These solvents are highly effective at removing not only fats but also some non-lipid components, including minerals that might be bound to lipids or proteins. As a result, the aggressive extraction process with these solvents may reduce the ash content in the final protein isolate. In the undefatted meal, some minerals may be bound to fats or proteins, limiting their availability. Water and 50% ethanol, being polar solvents, may have disrupted these bonds, released previously bound minerals and increased their availability, which then remain in the defatted protein isolate. The total solids content, representing the material remaining after moisture removal, was highest in 7EMGPI and lowest in WMGPI. A higher total solids content reflects a more concentrated protein isolate with minimal water content, enhancing stability and performance in food formulations (Liu et al., 2018). Conversely, the lower solids content in WMGPI suggests higher moisture retention, which may reduce shelf life and storage stability (Shen et al., 2021). The relationship between total solids and moisture is crucial for ensuring product stability. Higher moisture content, as seen in WMGPI, may promote microbial growth and spoilage, whereas higher dry matter content, as observed in 7EMGPI, ensures extended shelf life and suitability for diverse applications.

Table 3.3: Proximate composition (%) of protein isolates obtained from undefatted MS meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI).

Samples	Moisture	Crude protein	Crude fibre	Fat	Ash	Dry matter
EMGPI	1.89 ± 0.05^{c}	88.58 ± 0.20^{e}	0.04 ± 0.01^{bc}	2.02 ± 0.02^{b}	6.29 ± 0.06^{d}	98.12 ± 0.05^{c}
MMGPI	1.23 ± 0.01^{de}	96.95 ± 0.02^{a}	0.11 ± 0.02^{a}	0.50 ± 0.15^{d}	4.21 ± 0.01^{f}	98.48 ± 0.18^{bc}
HMGPI	1.19 ± 0.03^{de}	91.19 ± 0.12^{c}	0.07 ± 0.01^{b}	0.79 ± 0.08^{d}	5.54 ± 0.03^{e}	98.81 ± 0.03^{ab}
AMGPI	1.54 ± 0.02^{d}	89.39 ± 0.14^{d}	0.12 ± 0.01^{a}	1.31 ± 0.07^{c}	6.11 ± 0.00^{d}	98.47 ± 0.01^{bc}
UMGPI	1.48 ± 0.06^{d}	85.34 ± 0.11^{f}	0.04 ± 0.02^{bc}	1.42 ± 0.09^{c}	8.17 ± 0.21^{c}	98.52 ± 0.06^{bc}
7EMGPI	0.92 ± 0.02^{e}	95 ± 0.13^{b}	0.07 ± 0.01^{bc}	1.17 ± 0.02^{c}	5.58 ± 0.12^{e}	99.09 ± 0.02^{a}
5EMGPI	2.88 ± 0.31^{b}	84.36 ± 0.07^{g}	0.03 ± 0.00^{c}	3.62 ± 0.21^{a}	9.90 ± 0.01^{a}	97.18 ± 0.34^{d}
WMGPI	3.34 ± 0.01^{a}	88.35 ± 0.03^{e}	0.07 ± 0.01^{bc}	3.81 ± 0.10^{a}	8.57 ± 0.19^{b}	96.66 ± 0.01^{e}

The data represent the mean values of duplicate readings with their corresponding standard deviations. Different superscripts within the same column signify statistically significant differences among the samples ($p < 0.05$).

3.3.4 Amino acid composition

The amino acid profiles of MSPI are summarized in Table 3.4, showing significant variations across protein isolates. These profiles are crucial because they influence the protein's nutritional quality, functional properties, and biological activity (Day et al., 2022). The essential amino acids (EAA) content varies among the samples, with MMGPI showing the highest EAA content at 34.16%, while 5EMGPI has the lowest at 28.80%. The EAA profile of MSPI is similar to flaxseed protein isolate but lower than yellow mealworm larvae (44.93%) (Zhao et al., 2016) and hen's egg protein (43.6%) (Mishyna et al., 2019), while being higher than soy protein isolate (Kaushik et al., 2016). A high EAA content is particularly valuable for nutritional supplements aimed at supporting the health of low-birth-weight infants (Furuta et al., 2021). Consequently, MMGPI may be especially suitable for some infant foods. The lysine-to-arginine (Lys/Arg) ratio is a significant factor in assessing a protein's potential effects on cholesterol and cardiovascular health (Strauss et al., 2020). A lower Lys/Arg ratio is associated with reduced cholesterol levels and a reduced risk of atherosclerosis (Sanchez & Hubbard, 2018). This benefit is attributed to arginine's positive effects on cardiovascular health, such as improved blood vessel function and blood pressure reduction, whereas a high lysine intake relative to arginine has been linked to increased cholesterol and atherogenic effects (Venkatesh et al., 2017; Gambardella et al., 2020). In this study, all MSPIs showed similar Lys/Arg ratios, indicating comparable effects on lipid metabolism. WMGPI had the lowest Lys/Arg ratio at 0.10, suggesting a potentially favorable impact on cardiovascular health. In contrast, EMGPI showed the highest Lys/Arg ratio at 0.13, though still relatively low. These values are significantly lower than those observed in other plant-based protein isolates. For instance, flaxseed protein isolate has a Lys/Arg ratio of 0.22 (Marambe et al., 2008), and soy protein isolate has a much higher ratio of 0.71 (Kaushik et al., 2016). Given

the lower Lys/Arg ratios, MSPI may offer a health advantage in food formulations aimed at reducing lipid levels and atherogenic risks.

The total non-essential amino acids (NEAA) content across all samples exceeded the EAA content, with NEAA levels ranging from 40.84% (UMGPI) to 48.19% (MMGPI). This aligns with reports that plant proteins generally have higher proportions of NEAAs, such as Glu, Asp, Ala, and Gly, compared to EAAs like Lys, Met, and Try. This observation supports findings by Gorissen & Witard (2018), who indicated that plant-based proteins often have lower Met and Lys levels (Verzola et al., 2020). Among the NEAAs, Arg (8.80–10.76%) and Glu (14.43–16.83%) were the most abundant. Glu plays a key role in nitrogen metabolism and serves as a precursor for gamma-aminobutyric acid (GABA), a neurotransmitter (Sulieman et al., 2024). Thus, MSPI may be a rich source of Glu. NEAA levels in MSPI are similar to those found in *Moringa oleifera* leaves (Olaofe et al., 2013), lower than those of hen's egg (54.7%) and cow's milk (56.1%) (Mishyna, et al., 2019), but higher than *Moringa oleifera* seed cake powder (Patil et al., 2022).

Branched-chain amino acids (BCAA), including Leu, Ile, and Val, are essential for muscle repair and recovery. BCAA content ranged from 7.02% (5EMGPI) to 8.24% (MMGPI). In contrast, kidney bean vicilin reportedly has a higher BCAA content, around 18% (Mishyna, et al., 2019). Hydrophobic amino acids (HAA) were highest in MMGPI at 29.01% and lowest in UMGPI at 25.80%. HAA are commonly abundant in globular proteins, where they are often buried in the protein core or shielded by lipid layers (Bera et al., 2018). Solvents that interact with these proteins can influence the release or retention of HAA (Schwendeman et al., 2020). Methanol's efficiency as a solvent may have better exposed these hydrophobic regions, enhancing extraction. These findings are comparable to those for chickpea protein isolate (Ghribi et al., 2015), though lower than lentil seed protein isolate (Osemwota et al., 2021). Positively charged amino acids (PCAA)

like lysine and histidine are crucial for various biological functions. MMGPI had the highest PCAA levels, while UMGPI showed lower levels (2.89%). Solvent polarity affects amino acid retention by influencing lipid dissolution. High-polarity solvents like methanol and ethanol can dissolve polar lipids, potentially preserving polar amino acids like arginine and glutamic acid. Similarly, aromatic amino acids (AAA), such as phenylalanine and tryptophan, are better retained in solvents that minimize protein denaturation; for instance, methanol effectively preserves AAA.

The sulfur-containing amino acids (SCAA) content ranged from 2.06% in UMGPI to 2.85% in MMGPI. High SCAA levels enhance oxidative stability, as sulfur-containing amino acids have antioxidant properties that help combat oxidative stress (Famuwagun et al., 2021). Although the relatively low SCAA levels are typical for plant proteins, Met and Cys are crucial for growth, cellular repair, and metabolism. Met is essential as a methyl donor in biochemical reactions, while Cys is a key component of the antioxidant glutathione (Kachungwa et al., 2022). Notably, 7EMGPI exhibited the highest Cys content at 1.30%, a value comparable to soy protein isolate, higher than buckwheat protein isolate, but lower than rice protein isolate (Tang & Wang, 2010; Jin et al., 2021). Among all samples, MMGPI displayed the highest concentrations across various amino acid groups, including EAAs, BCAAs, and SCAAs, indicating methanol's effectiveness in preserving amino acids during defatting. However, it is worth noting that 7EMGPI shows comparable values to MMGPI for most amino acid groups. This suggests that 7EMGPI may also retain a significant amino acid profile, making it a competitive option in terms of amino acid preservation and nutritional quality.

In contrast, UMGPI, 5EMGPI, and WMGPI exhibited comparably lower amino acid content across most groups. The low values in UMGPI suggest that the defatting process may contribute to the concentration of amino acid residues, as seen in the higher amino acid levels in

the defatted samples. This indicates that defatting not only removes non-protein components but may also enhance the relative abundance of amino acids, thereby improving the protein quality and nutritional value in these defatted samples. However, the relatively low amino acid contents in 5EMGPI and WMGPI indicate that certain solvents, such as water and 50% ethanol, may be less efficient in protein extraction or may result in amino acid loss. The observed variations in amino acid composition across different solvent treatments highlight the importance of selecting appropriate defatting methods to maximize nutritional value.

Table 3.4: Amino acid composition (%) of protein isolates obtained from undefatted *Moringa stenopetala* meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI).

Amino acids	EMGPI	MMGPI	HMGPI	AMGPI	UMGPI	7EMGPI	5EMGPI	WMGPI
Amm	1.15 ± 0.05	1.31 ± 0.02	1.26 ± 0.02	1.18 ± 0.05	1.16 ± 0.04	1.32 ± 0.04	1.10 ± 0.02	1.11 ± 0.05
His	1.64 ± 0.09	1.74 ± 0.04	1.60 ± 0.11	1.70 ± 0.02	1.53 ± 0.12	1.68 ± 0.00	1.54 ± 0.04	1.81 ± 0.11
Ser	1.94 ± 0.01	2.15 ± 0.01	2.04 ± 0.06	1.91 ± 0.03	1.85 ± 0.00	2.06 ± 0.02	1.81 ± 0.05	1.84 ± 0.01
Arg	9.50 ± 0.09	10.76 ± 0.09	10.07 ± 0.40	9.77 ± 0.15	9.14 ± 0.21	10.59 ± 0.20	8.80 ± 0.15	10.37 ± 0.01
Gly	2.76 ± 0.00	2.98 ± 0.02	2.90 ± 0.10	2.80 ± 0.02	2.62 ± 0.06	2.91 ± 0.05	2.72 ± 0.06	2.62 ± 0.04
Asp	4.36 ± 0.01	4.75 ± 0.01	4.57 ± 0.12	4.18 ± 0.09	4.13 ± 0.02	4.57 ± 0.00	4.24 ± 0.13	4.06 ± 0.11
Glu	15.07 ± 0.00	16.83 ± 0.12	15.92 ± 0.52	15.12 ± 0.02	14.43 ± 0.19	16.80 ± 0.05	14.51 ± 0.37	15.28 ± 0.03
Thr	1.87 ± 0.02	2.03 ± 0.01	1.94 ± 0.07	1.87 ± 0.01	1.74 ± 0.05	1.97 ± 0.04	1.78 ± 0.03	1.87 ± 0.01

Ala	2.84 ± 0.00	3.09 ± 0.22	3.00 ± 0.08	2.83 ± 0.03	2.71 ± 0.03	2.98 ± 0.03	2.68 ± 0.06	2.83 ± 0.01
Pro	3.95 ± 0.06	4.71 ± 0.53	4.25 ± 0.14	3.98 ± 0.00	3.74 ± 0.07	4.24 ± 0.06	3.67 ± 0.08	3.84 ± 0.06
Cys	1.00 ± 0.05	1.26 ± 0.05	0.95 ± 0.06	1.03 ± 0.01	0.81 ± 0.03	1.30 ± 0.00	1.09 ± 0.05	1.30 ± 0.04
Lys	1.25 ± 0.04	1.34 ± 0.04	1.28 ± 0.04	1.20 ± 0.03	1.18 ± 0.02	1.22 ± 0.01	1.09 ± 0.01	1.09 ± 0.05
Tyr	1.47 ± 0.01	1.67 ± 0.01	1.59 ± 0.08	1.52 ± 0.01	1.41 ± 0.04	1.60 ± 0.06	1.46 ± 0.03	1.47 ± 0.05
Met	1.43 ± 0.07	1.59 ± 0.01	1.41 ± 0.05	1.41 ± 0.04	1.25 ± 0.04	1.48 ± 0.03	1.30 ± 0.03	1.37 ± 0.07
Val	3.43 ± 0.01	3.82 ± 0.02	3.65 ± 0.13	3.46 ± 0.02	3.27 ± 0.01	3.67 ± 0.06	3.28 ± 0.06	3.28 ± 0.03
Ile	2.59 ± 0.05	2.82 ± 0.04	2.76 ± 0.09	2.58 ± 0.02	2.48 ± 0.00	2.71 ± 0.05	2.39 ± 0.06	2.57 ± 0.01
Leu	4.89 ± 0.03	5.42 ± 0.05	5.19 ± 0.19	4.96 ± 0.04	4.66 ± 0.08	5.22 ± 0.07	4.63 ± 0.12	5.00 ± 0.05
Phe	3.28 ± 0.04	3.60 ± 0.02	3.46 ± 0.13	3.31 ± 0.08	3.15 ± 0.06	3.40 ± 0.09	3.11 ± 0.07	3.25 ± 0.06
Trp	0.92 ± 0.03	1.04 ± 0.00	0.97 ± 0.00	0.95 ± 0.01	0.93 ± 0.00	0.93 ± 0.01	0.90 ± 0.03	0.93 ± 0.03
AAA	5.68	6.31	6.01	5.78	5.59	5.92	5.46	5.65

BCAA	7.49	8.24	7.95	7.53	7.15	2.71	7.02	7.57
HAA	25.80	29.01	27.22	26.03	24.42	27.51	24.48	25.84
PCAA	2.89	3.08	2.88	2.90	2.71	2.90	2.64	2.90
NCAA	19.43	21.58	20.49	19.30	18.56	21.38	18.74	19.34
SCAA	2.42	2.85	2.36	2.44	2.06	2.78	2.39	2.67
EAA	30.82	34.16	32.30	31.20	29.32	32.87	28.80	31.53
NEAA	42.88	48.19	45.28	43.15	40.84	47.05	40.97	43.60

HAA - hydrophobic amino acids (alanine (Ala), valine (Val), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), tryptophan (Try), proline (Pro), methionine (Met), and cysteine (Cys)), PCAA - positively charged amino acids (histidine (His), lysine (Lys)), NCAA- negatively charged amino acids (ASP - asparagine + aspartic acid) and (GLU - glutamine + glutamic acid), AAA - aromatic amino acids (phenylalanine, tryptophan, and tyrosine), SCAA - sulphur containing amino acids (cysteine and methionine), BCAA - branched chain amino acids (leucine and isoleucine), EAA - essential amino acids (threonine (Thr), valine, methionine, isoleucine, leucine, phenylalanine, histidine, lysine, arginine (Arg), and tryptophan), NEAA – non-essential amino acids (alanine, arginine, ASP (asparagine + aspartic acid), cysteine, GLU (glutamic acid + glutamine), glycine (Gly), proline, serine (Ser), and tyrosine).

The data represent the mean values of duplicate readings with their corresponding standard deviations. Different superscripts within the same column signify statistically significant differences among the samples ($p < 0.05$).

3.3.5 Total polyphenol content

Polyphenol content in foods is crucial for understanding their bioactivity, functionality, and potential health benefits (Rana et al., 2022). Monitoring these levels helps optimize formulations without compromising bioactivity (Gunasekaran et al., 2014). The total polyphenol content (TPC) of MSPI, as shown in Table 3.5, varied significantly depending on the defatting methods employed during sample preparation. For TPC determination, polyphenols were extracted using specific solvent systems, including 80% methanol, combination of 80% and 50% methanol, and another combination of methanol (80% and 50%) with 70% acetone. UMGPI exhibited the highest TPC across all defatting solvents, with values ranging from 1.39 to 1.53 mg GAE/g for the 80% methanol extraction, indicating that defatting significantly ($p < 0.05$) reduced the level of polyphenols. In contrast, WMGPI recorded the lowest polyphenol content across most of the extracting solvents, except for 80% methanol, where 7EMGPI had the lowest value, followed closely by WMGPI. This suggests that 70% ethanol caused a substantial loss of polyphenols during defatting. Similarly, WMGPI's low polyphenol levels can be attributed to water's high solubility for polyphenols, resulting in their removal during water-based defatting. This aligns with Jones et al. (2006), who reported that polyphenols from broccoli tissue were lost primarily through leaching into water, though the extent of loss depended on water volume.

TPC was generally highest with 80% methanol extraction, demonstrating the superior ability of this solvent to recover polyphenols from protein isolates. In contrast, the methanol-acetone system yielded lower polyphenol values, indicating lower efficiency. For instance, WMGPI dropped from 1.29 mg GAE/g in 80% methanol to 0.57 mg GAE/g with the methanol-acetone combination. These findings highlight the significant influence of defatting methods and extraction solvents on the polyphenol content of MSPI. The defatting process can lead to the loss

of polyphenols, which are valued for their antioxidant and health-promoting properties. Ethanol-based methods appeared to better preserve polyphenols compared to other solvents. Among the extraction solvents, 80% methanol proved most effective in recovering polyphenols. The TPC results from this study are more closely aligned with the values reported for pumpkin seed, rice, and pea protein isolates. However, a higher TPC was observed for hemp protein isolates (Sawicki et al., 2024). The variation in TPC among these protein isolates may be attributed to differences in the raw material's polyphenol composition, processing conditions, and the efficiency of polyphenol retention during protein isolation.

Table 3.5: Functional and physicochemical properties of protein isolates obtained from undefatted *Moringa stenopetala* meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI).

Sample		UMGPI	AMGPI	HMGPI	EMGPI	MMGPI	WMGPI	7EMGPI	5EMGPI
TPC (mg GAE/g)	8M	1.53±1.73^{a}	1.36±3.33^{d}	1.36±3.01^{e}	1.47±3.85^{b}	1.38±5.77^{c}	1.29±5.09^{f}	1.08±1.92^{g}	1.36±4.19^{d}
	8,5M	1.44±1.73^{a}	1.13±3.42^{d}	1.33±5.09^{c}	1.38±1.93^{b}	1.11±1.84^{e}	0.73±3.33^{h}	0.95±1.92^{g}	1.00±3.33^{f}
	8,5M,7A	1.39±1.93^{a}	1.01±3.67^{d}	1.24±1.93^{b}	1.20±5.09^{c}	0.94±0.19^{f}	0.57±3.85^{h}	0.92±1.76^{g}	0.98±2.27^{e}
BI		23.77±0.11^{f}	28.24±0.23^{a}	23.57±0.04^{f}	26.79±0.1^{c}	23.34±0.04^{fg}	24.2±0.1^{e}	27.85±0.06^{b}	26.67±0.07^{cd}
SH		13.61±1.06^{cd}	22.94±0.07^{b}	20.05±2.05^{b}	18.31±0.21^{bc}	32.67±1.89^{a}	12.37±1.07^{d}	23.56±0.46^{b}	6.11±0.40^{e}
DSC	Tθ (^{o}C)	85.06±0.20^{g}	85.00±0.20^{h}	85.52±0.26^{c}	85.20±0.38^{f}	85.28±0.06^{d}	85.88±0.33^{a}	85.21±0.00e	85.57±0.34^{b}
	T_p (^{o}C)	88.77±0.07^{c}	88.77±0.17^{c}	89.19±0.36^{b}	88.72±0.01^{d}	88.67±0.01^{e}	89.18±0.52^{b}	88.75±0.02^{cd}	89.66±0.45^{a}
	ΔH (J/g)	0.24 ± 0.02^{b}	0.25 ± 0.03^{a}	0.22 ± 0.02^{c}	0.23 ± 0.04^{b}	0.21 ± 0.05^{d}	0.10 ± 0.00^{f}	0.21 ± 0.01^{d}	0.16 ± 0.01^{e}
WHC (g/g)		2.15±0.11^{d}	2.25±0.11^{cd}	2.52±0.06^{b}	2.25±0.14^{cd}	2.20±0.06^{cd}	2.49±0.11^{b}	2.28±0.03^{c}	2.81±0.07^{a}
OHC (g/g)		3.2±0.18^{d}	3.73±0.08^{ab}	3.64±0.17^{bc}	3.00±0.00^{d}	2.98±0.14^{d}	3.47±0.02^{c}	3.93±0.37^{a}	3.78±0.22^{ab}

IVPD (%)	76.52±0.36^{ef}	78.69±0.18^{c}	76.97±0.45^{e}	78.96±0.63^{c}	80.14±0.54^{b}	77.24±0.54^{de}	81.32±0.27^{a}	77.88±0.91^{d}

Total polyphenol content (TPC), bitterness intensity (BI), surface hydrophobicity (SH), differential scanning calorimetry (DSC), water holding capacity (WHC), oil holding capacity (OHC), in_vitro protein digestibility (IVPD), 8M (80 % methanol), 8,5M (80 % methanol, 50 % methanol), 8,5M,7A (80 % methanol, 50 % methanol, 70 % acetone), T_0 (^{0}C) (onset temperature), and Tp (^{0}C) (maximum temperature), ΔH (J/g) (enthalpy change). The data represent the mean values of duplicate readings with their corresponding standard deviations. Different superscripts within the same row signify statistically significant differences among the samples ($p < 0.05$).

3.3.6 E-tongue testing

Bitterness is a critical sensory attribute that can significantly influence the acceptability of food products, especially when protein isolates are used in formulations. Compounds such as saponins, alkaloids, glucosinolates, and polyphenols are primarily responsible for the bitterness in Moringa (Valdés-Rodríguez, 2023). However, Kashyap et al. (2022) highlighted that glucosinolates are the primary contributors to Moringa's bitter taste. In this study, the bitterness levels of MSPI varied notably, as shown in Table 3.5. The original undefatted MS seed meal flour exhibited a notably low bitterness intensity of 18.02, probably because of interference of lipids with solubilization of the bitter-tasting compounds. In contrast, the bitterness intensity of the defatted protein isolates ranged from 23.34 to 28.24, significantly higher than the soybean protein hydrolysate's intensity of 5.1 (Tong et al., 2020). Among the defatted samples, the isolate with the lowest bitterness intensity was still comparable to Denatonium Benzoate, a standard known for its extremely bitter taste, with a value of 23. The highest bitterness intensity was observed in the AMGPI. MMGPI had the lowest bitterness intensity at 23.34, which showed no significant difference from HMGPI and UMGPI. This suggests that both defatting methods and the protein isolation process contribute to the concentration of bitter compounds in MSPI.

The elevated bitterness in AMGPI could be due to acetone’s high efficiency in removing lipids, which increases the exposure of bitter compounds such as glucosinolates. Acetone may also disrupt the lipid-protein matrix, making these bitter compounds more accessible and intensifying the overall bitterness. Both 7EMGPI and EMGPI exhibited high bitterness intensity of 27.85 and 26.79, respectively. WMGPI, on the other hand, exhibited a moderate bitterness score. This could be attributed to water's selective solubility for hydrophilic bitter compounds, such as alkaloids, saponins, and polyphenols, which readily dissolve in water. This is consistent with WMGPI’s

lowest recorded total phenol content, as phenolics often contribute to bitterness. Glucosinolates, which are inherently water-soluble, may remain in the isolate if they are not fully hydrolyzed. Upon hydrolysis, glucosinolates break down into products such as isothiocyanates, thiocyanates, and nitriles, which also contribute to flavor and bitterness depending on their concentrations (Bell et al., 2018). The partial removal of certain bitter compounds could explain the moderate bitterness observed in WMGPI. In contrast, 7EMGPI displayed a high bitterness intensity despite its lower total phenol content, suggesting that polyphenol alone may not account for the bitterness. Other compounds, such as specific amino acids or peptides, may also contribute to the observed bitterness. This indicates that the overall composition, including both the amino acid profile and polyphenol content, may interact to influence the sensory characteristics like bitterness (Osakabe et al., 2024).

Overall, the results indicate that the defatting method intensifies the concentration of bitter-tasting compounds in MSPI. The protein isolation process itself likely contributes to this effect, as some proteins may inherently possess a bitter taste. It is also possible that during protein isolation, the bitter taste compounds could bind to or interact with proteins, further enhancing the bitterness of the final product. These findings suggest that both the concentrated bitter compounds and the proteins themselves play a role in the overall bitter taste profile of the isolates. Moreover, acetone and ethanol (including 70% ethanol) defatting methods appear to significantly increase the bitterness of MSPI due to the higher exposure to bitter-tasting compounds.

3.3.7 Surface hydrophobicity

Surface hydrophobicity reflects the extent to which hydrophobic amino acid residues are exposed on the protein's surface (Zhu et al., 2016). This property provides key insights into functional characteristics such as solubility, emulsification, and foaming capacity (Yan et al.,

2021). Protein unfolding exposes hydrophobic residues that were previously buried within the protein's core (Bassogog et al., 2022). Table 3.5 presents the surface hydrophobicity of MSPI. The defatted samples showed significant ($p < 0.05$) variation in surface hydrophobicity, indicating that the solvent type influences the exposure of hydrophobic residues. Among the defatted samples, MMGPI exhibited the highest hydrophobicity (32.67), suggesting that methanol promotes greater unfolding and exposes more hydrophobic residues. In contrast, 5EMGPI had the lowest hydrophobicity (6.11), implying better preservation of protein structure with limited exposure of hydrophobic groups. Similarly, WMGPI showed relatively low hydrophobicity, comparable to the undefatted sample. This suggests that water defatting causes less structural disruption than organic solvents, resulting in fewer exposed hydrophobic residues. As a result, these proteins may have reduced capacity to stabilize oil-water interfaces in emulsions. On the other hand, 7EMGPI recorded moderate hydrophobicity with higher value next to MMGPI, likely reflecting a mild denaturing effect that partially unfolds the protein, exposing some hydrophobic regions while retaining structural integrity. The higher hydrophobicity observed in MMGPI and 7EMGPI is consistent with the greater exposure of hydrophobic amino acid residues in these samples. These findings align with the observations of Aider et al. (2012), who reported that greater surface hydrophobicity reflects a higher abundance of hydrophobic amino acids.

The surface hydrophobicity values observed in this study are lower than those reported for Moringa oleifera seed protein isolates, which recorded 50.38 (Aderinola et al., 2020. The variation in hydrophobicity across treatments underscores the role of defatting and solvent choice in modulating protein conformation and the extent of hydrophobic residue exposure. Higher hydrophobicity generally enhances protein-protein interactions, which can influence emulsifying and foaming properties (Chao & Aluko, 2018). Given its high hydrophobicity, MMGPI and

7EMGPI shows potential for use in formulations requiring strong emulsifying agents. These findings support previous research suggesting that organic solvents can alter protein structures in ways that either increase or decrease hydrophobic interactions, depending on the solvent's polarity and interaction with the protein matrix (Sanchez-Fernandez and Jackson, 2021).

3.3.8 Differential scanning calorimetry

Table 3.5 presents the thermal properties of MSPI, highlighting onset temperature (Tθ), denaturation temperature (Tp), and enthalpy change (ΔH). Tθ indicates the initial phase of protein denaturation (Basharov, 2012). In this study, Tθ values for MSPI samples vary slightly, ranging from 85.00°C to 85.88°C. Notably, WMGPI show the highest onset temperatures, suggesting that these proteins showed more resistance to initial thermal denaturation. The likely presence of retained water in WMGPI may stabilize its structure through hydration, thereby mitigating thermal stress (Bilal et al., 2021). Conversely, AMGPI has the lowest Tθ. The Tθ values observed here surpass the 66.50°C reported for pea protein concentrate (Asen & Aluko, 2022) but align closely with values reported for lentil protein isolates (Osemwota et al., 2021). The Tp denotes the peak rate of denaturation (Liu et al., 2014). 5EMGPI exhibited the highest Tp values (89.66 ^{o}C) indicating higher thermal stability while MMGPI had comparatively lower Tp value (88.67 ^{o}C), suggesting less thermal stability. These Tp values remain lower than the 94.30–95.05°C reported for hemp seed protein isolates (Fang et al., 2023), underscoring variability in thermal tolerance across protein type. ΔH reveals the energy necessary for denaturation, directly correlating with structural stability (Seelig & Seelig, 2023). ΔH values for MSPI range between 0.10 J/g and 0.25 J/g, which is significantly ($p < 0.05$) lower than the 11.4 J/g that was reported for buckwheat globulin (Tang & Wang, 2010) and 75.93 J/g for chickpea protein isolate (Ghribi et al., 2015) but slightly higher than the 0.16 J/g for lentil protein isolates (Miranda et al., 2022). WMGPI with the

lowest ΔH, might possess fewer stabilizing interactions, implying reduced structural stability. Meanwhile, AMGPI displayed the highest ΔH value. Alabi (2023) reported that ΔH suggests the degree of undenatured proteins or the extent of ordered structure. The ΔH values, particularly for AMGPI, reinforce that these proteins may retain more ordered structures under thermal stress. Higher Tp and ΔH values generally enhance a protein's suitability for heat-stable applications, making the proteins potentially advantageous in food processing or nutraceutical formulations where stability under heat is desired (Dias et al., 2021).

3.3.9 Water and Oil holding capacity

The oil-holding capacity (OHC) and water-holding capacity (WHC) are critical functional properties of protein isolates, reflecting their ability to retain oils and water in food systems (Zakki et al., 2024). These properties are essential for applications like emulsification, texture enhancement, and moisture retention in various food formulations (Ma et al., 2022). Table 3.5 summarizes the OHC and WHC of the MSPI. The OHC values ranged from 2.98 to 3.93 g/g, showing significant variation ($p < 0.05$) across treatments. Among the samples, 7EMGPI exhibited the highest OHC, suggesting that the ethanol defatting of the MS meal may have partially denatured the proteins to expose hydrophobic groups, which contributed to enhanced affinity for oil. In contrast, MMGPI recorded the lowest OHC, which suggest a protein surface with less hydrophobic groups than the other protein isolates. This aligns with observations from prior studies, where increased exposure of hydrophobic amino acid residues tends to boost OHC (Aderinola et al., 2020). However, despite having only the second-highest hydrophobic amino acid content, 7EMGPI exhibited the highest OHC, indicating that factors beyond hydrophobicity such as structural conformation and physical characteristics like bulk density might also contribute. For example, Joshi et al. (2015) noted that higher bulk density can improve OHC by increasing oil

absorption sites. OHC plays a vital role in new product development, enhancing flavor retention and slowing lipid oxidation, which delays rancidity (Zhang et al., 2024). High OHC also stabilizes fat-rich foods, extending shelf life and improving product quality (Aderinola et al., 2020). Notably, the OHC values for MSPI are lower than those reported for pea protein isolates (Asen & Aluko, 2022).

WHC values ranged from 2.15 to 2.81 g/g, indicating the proteins' ability to retain water. The highest WHC was observed in 5EMGPI, suggesting that the moderate ethanol concentration pretreatment of the MS meal enhanced water retention by favourable altering the protein structure. This could also be linked to its solubility, since samples with lower protein solubility exhibit higher WHC (Rezaei et al., 2022). HMGPI and WMGPI also showed relatively high WHC, while UMGPI exhibited the lowest WHC. This suggests that defatting improves WHC by restructuring the protein, exposing hydrophilic regions that enhance water-binding capacity (Nahimana et al., 2023). Similarly, egg yolk hydrolysates reported comparable behavior (Bao et al., 2017). However, the OHC and WHC of the MSPI are lower than those of sesame seed protein isolates (Rezaei et al., 2022). Interestingly, albumin fractions in *Moringa oleifera* seed proteins showed no WHC, likely due to their high solubility from aqueous extraction (Aderinola et al., 2020).

The results demonstrate that the defatting method significantly influenced the OHC and WHC of proteins isolated from the defatted meal. In this study, the results from 7EMGPI and 5EMGPI, isolated from ethanol-defatted MS meal, suggest that the ethanol concentration used for defatting significantly impacted both the oil and water retention properties of the proteins. The 7EMGPI demonstrated superior oil retention, likely due to mild protein denaturation that exposed hydrophobic regions, facilitating stronger oil binding. In contrast, the 5EMGPI showed superior water retention, indicating that a 50% ethanol treatment may have enhanced the exposure of

hydrophilic regions, optimizing water binding. These findings suggest that the ethanol concentration during defatting can be adjusted to modulate protein structural changes that optimize oil and water retention. For example, Adhikari et al. (2022) also reported that hydrophobic residues enhance oil binding, while exposed hydrophilic regions promote water retention. In contrast, the lower OHC and WHC values observed for proteins isolated from the undefatted MS meal (UMGPI) suggest that native lipids may have interfered with additional interactions with water or oil. These differences across the protein isolates from different defatted meals highlight the potential for tailoring protein functionality for specific food applications. For instance, 7EMGPI and 5EMGPI, with their high OHC and WHC, would be ideal for products requiring both moisture retention and fat stabilization, such as baked goods or emulsions. Conversely, isolates like UMGPI and MMGPI may be better suited for dry powders or snacks, where low oil and water retention is advantageous.

3.3.10 In vitro protein digestibility (IVPD)

Table 3.5 presents IVPD of the MSPI, which reflects how easily the proteins can be broken down during digestion, which is an essential factor in evaluating their bioavailability and overall nutritional quality. The IVPD ranged from 76.52% to 81.32%, aligning with previous findings for roasted, debittered Moringa peregrina press cake, green lentil protein concentrate, and pea protein concentrate (Barbana & Boye, 2013; Çabuk et al., 2018; Sardabi et al., 2022). However, these values are lower than those reported for Kariya protein isolate and black and yellow quinoa protein isolates (Adiamo et al., 2016; Sánchez-Reséndiz et al., 2019). Among the samples, 7EMGPI exhibited the highest digestibility, while UMGPI had the lowest. This suggests that defatting of the meal enhanced digestibility of subsequently isolated proteins, likely because lipids and other non-protein components present in undefatted sample can interfere with enzymatic activity or

reduce accessibility. The variation in digestibility across samples indicates that the choice of defatting solvent plays a critical role. For instance, HMGPI showed lower digestibility compared to other defatted samples, possibly because hexane may cause partial protein denaturation and aggregation that led to impaired enzymatic digestion. High digestibility is advantageous for formulating protein supplements or functional foods aimed at enhancing protein intake and absorption, particularly for consumers requiring easily digestible plant-based proteins (Kuesten & Hu, 2020).

3.3.11 Intrinsic fluorescence

The intrinsic fluorescence analysis of MSPI across varying pH levels (3, 5, 7, and 9) reveals structural shifts and changes in the exposure of aromatic amino acids, notably tryptophan and tyrosine, as shown in Figure 3.4. Fluorescence intensity, which depends on the environment of these aromatic residues, reflects tertiary structural alterations in the protein at different pH conditions (Wang et al., 2017). The spectra indicate a weak tyrosine peak at 303 nm and a more prominent tryptophan peak at 348 nm. The weak tyrosine signal, particularly at pH 3, may be due to its low quantum yield, contrasting with the strong emission of tryptophan. This suggests low surface exposure or minimal contribution, which may indicate either highly exposed tyrosine in a hydrophilic environment (Aderinola et al., 2020) or enhanced energy transfer from tyrosine to tryptophan due to strong protein-protein interactions (Malomo & Aluko, 2015). Similar to this study, previous reports have shown λmax for tyrosine emission below 320 nm in *Moringa oleifera* seed protein isolate (Aderinola et al., 2020), hemp seed protein isolate (Malomo & Aluko, 2015), and resveratrol in composite nanoparticles (Wei et al., 2019).

The tryptophan peak is consistently detectable across all pH levels, with the highest intensity observed at pH 3. This elevated intensity suggests reduced exposure of hydrophobic

amino acids to the hydrophilic environment and high degree of protein folding at this pH (Agboola & Aluko, 2009). As the protein transitions from a compact, folded state to a more exposed, unfolded form, tryptophan emission may shift toward shorter longer wavelengths, leading to decreases in fluorescence intensity (Schmid, 1990; Wei et al., 2019). At pH 5, near the isoelectric point of MSPI, tryptophan fluorescence intensity decreases, likely due to protein aggregation; reduced charge repulsion at this pH facilitates aggregation and enhances hydrophilic interactions. A further decrease in tryptophan intensity at pH 7 corresponds with another dip in MSPI solubility. At pH 9, tryptophan intensity slightly increases, as the protein moves farther from its isoelectric point.

The lower fluorescence intensities observed at pH 5, 7, and 9 suggest a less compact protein structure, where aromatic and hydrophobic residues are far apart and engaged in more interactions with the hydrophilic environment (Du et al., 2022). Interestingly, as tryptophan intensity decreases across pH 5, 7, and 9, tyrosine intensity increases relative to its low level at pH 3, possibly indicating spatial separation from tryptophan residues (Jones & Farrens, 2014). Notable differences in fluorescence intensity are observed among the isolates. WMGPI showed the lowest intensity across most pH levels, except at pH 9 where 7EMGPI has the lowest intensity. This indicates that water and 70% ethanol defatting may have resulted in a less compact structure where aromatic and hydrophobic amino acids are exposed to the hydrophilic environment. This reduced compact conformation could explain the lower intensity, as fluorescence is generally weaker when aromatic residues like tryptophan are more exposed to the solvent environment. Meanwhile, MMGPI exhibited the highest intensities at most pH values, possibly due to its higher hydrophobic and aromatic amino acid content. This result suggest that methanol induces a compact, more folded protein structure that allows aromatic residues to interact more readily with each other in the

hydrophobic core of the protein and away from the hydrophilic environment. The results from this study are consistent with the observations reported by Yin et al. (2011) and Malomo & Aluko (2015), who showed that the intensities of tryptophan and tyrosine residues vary significantly with changes in buffer pH from 3.0 to 9.0. This indicates that the microenvironment surrounding aromatic residues is highly sensitive to pH conditions. These findings further reinforce that protein structural conformations are affected by environmental factors, which influence folding behavior and the exposure of aromatic groups.

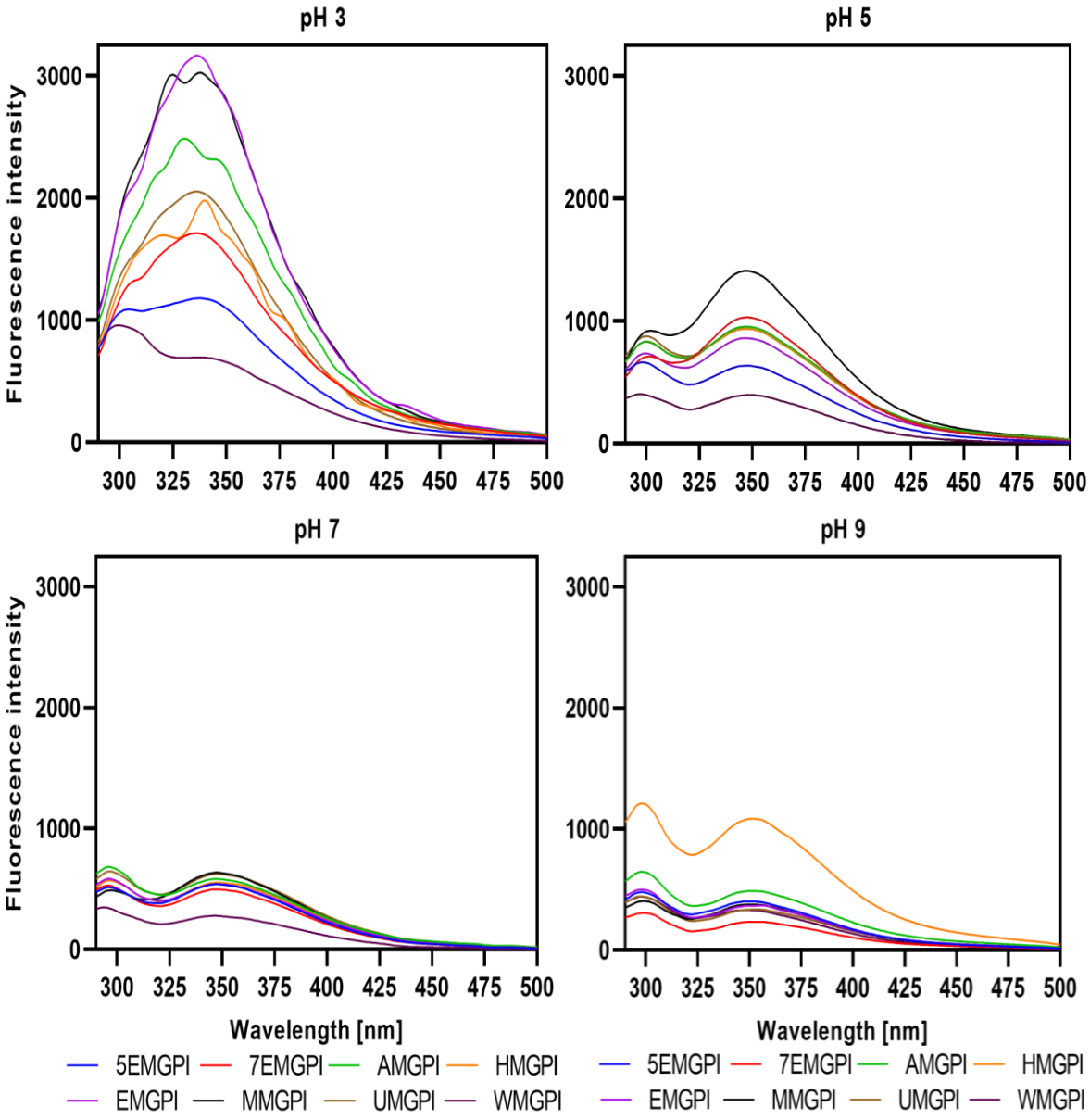


Figure 3. 4: Intrinsic fluorescence of protein isolates obtained from undefatted MS meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI).

3.3.12 Circular dichroism (secondary and tertiary structures)

Figure 3.5 displays the near-UV circular dichroism (CD) spectra for MSPI analyzed at different pH levels (3, 5, 7, and 9). Near-UV CD primarily provides information on the tertiary structure of proteins, especially focusing on the environment around aromatic amino acids like phenylalanine, tyrosine, and tryptophan, as well as disulfide bonds, which contribute to the folding and overall structural stability of proteins (Linhares & Ramos, 2023). The spectra show significant variations in mean residue ellipticity (MRE) across the isolates, with notable negative ellipticities observed around 260–280 nm. The absorbance at approximately 260 nm indicates the presence of phenylalanine residues, while the absorbance around 280 nm corresponds to tryptophan residues. This observation is consistent with prior studies on Moringa oleifera seed protein isolates (Aderinola et al., 2020). Additionally, peaks observed near 250 nm may be attributed to disulfide bonds, as minor CD signals in this region are known to arise from these structural elements (Villanueva et al., 2024).

At pH 3, many samples exhibited pronounced ellipticities in the 260-320 nm range, suggesting a relatively structured environment around aromatic residues and a more ordered tertiary structure. This is consistent with the findings of Schmid (1990), who reported that acidic conditions stabilize protein structures, possibly through enhanced hydrogen bonding and charge interactions. Notably, EMGPI, HMGPI, and AMGPI show higher negative ellipticity at this pH, implying more ordered or stable tertiary structures. A stronger negative ellipticity often correlates with a higher degree of ordered secondary structures, such as α-helices or β-sheets, as described by Lopes et al. (2018) and Kuril et al. (2024). At pH 5, the overall negative ellipticity is slightly reduced compared to pH 3, with EMGPI and MMGPI showing the highest negative ellipticity values. This decrease in ellipticity suggests mild structural rearrangements or unfolding,

potentially due to the proximity of pH 5 to the isoelectric point, where electrostatic repulsion is minimized, leading to reduced structural stability. However, these findings contradict Malomo & Aluko (2015), who reported an undetectable CD spectrum for hemp seed protein isolate at this pH, likely due to protein insolubility. At pH 7 and 9, the ellipticity values approach zero for most samples, indicating a predominantly disordered or unfolded protein conformation. Lower ellipticity at these pH levels suggests a loss of tertiary structure stability, with the protein shifting toward a more random coil or unordered conformation (Batumalaie et al., 2018). This interpretation aligns with Fehér et al. (2020), who noted that near-zero ellipticity is associated with minimal ordered secondary structures, such as α-helices and β-sheets, which are typically lost under these conditions. The flatter spectra, especially at pH 9, indicate a high degree of denaturation and structural rearrangement, with aromatic residues becoming more exposed to the hydrophilic environment as the tertiary structure destabilizes (Alabi, 2023). Adhav & Saikrishnan (2023) also explains that higher pH levels can disrupt the non-covalent interactions (like hydrogen bonds and ionic interactions) that help maintain the protein's folded state, leading to structural rearrangement or denaturation.

The defatting method significantly impacts the structural stability of MSPI. AMGPI and HMGPI demonstrate more pronounced negative ellipticity at acidic pH levels, particularly at pH 3, suggesting better-preserved tertiary structures. However, at higher pH values, the spectra for these isolates converge and show reduced ellipticity, indicating that these isolates may be more stable at lower pH but denature more readily in basic conditions. In contrast, WMGPI consistently shows the lowest ellipticity across all pH levels, indicating a less ordered, more flexible, or partially unfolded structure. Lower ellipticity typically corresponds to a more disordered protein environment (Baxa et al., 2024), suggesting that water-based defatting may lead to greater protein

denaturation or destabilization. Stollar & Smith (2020) explain that this reduced structural integrity can affect the protein's functionality and stability.

The far-UV CD spectra are essential for understanding the secondary structure of proteins, providing insights into protein degradation and denaturation (Ruzza et al., 2021). Structural changes can impact the protein's functional properties, including solubility, digestibility, and bioactivity (Sun, 2024). In Table 3.6, the secondary structure of MSPI is predominantly unordered, followed by β-sheets and β-turns. This trend is similar to what Sun et al. (2024) reported for dried fish protein, suggesting that the processing of MSPI likely induced unfolding and structural rearrangements. The low α-helix content, consistently below 10% across most samples, suggests partial or complete denaturation. This is indicative of solvent interactions during defatting or pH-induced unfolding, with α-helices, generally being more stable in native proteins, lost during the isolation process. However, an exception is noted for MMGPI, which displays a significantly higher α-helix content (up to 23.05%) at pH 3. A similar increase in α-helix content at low pH was reported by Jeong et al. (2021) for *Gryllus bimaculatus* protein concentrate, suggesting that specific extraction methods and conditions may help preserve helical structures to a degree. Compared to previous findings, the α-helix content here is notably lower than reported by Choi & Ma (2007) for buckwheat globulin and by Tang and Wang (2010) for other plant proteins. This pH sensitivity aligns with the observed near-UV spectra, where increased ellipticity at lower pH suggests less structural disruption. The β-sheet content exhibits considerable variation depending on pH and defatting treatment, with its lowest value often seen at pH 7. Given that β-sheets contribute significantly to protein stability, this variability may reflect structural rearrangements where β-sheets decrease as α-helices are lost. At pH 7, the reduced β-sheet content likely results from conformational shifts that favor unordered structures. This pattern is reflected in the near-

UV CD spectra, where ellipticity tends to decrease around pH 7, indicating greater tertiary structure disorder.

β-turns, which contribute to protein flexibility, remain relatively stable across pH levels and defatting methods, typically between 14% and 19%. Shapovalov et al. (2019) similarly reported that while β-turns are common, they do not dominate protein structure. High levels of unordered structure are observed in all samples, particularly in MMGPI and 7EMGPI. The unordered structure tends to increase at pH 7 and 9, likely due to further unfolding or denaturation as pH deviates from the isoelectric point. This trend is consistent with the near-UV spectra, where samples show lower ellipticity at these pH levels, reflecting a more disordered tertiary structure. Mune Mune et al. (2017) found that increased unordered structures often correlate with higher surface hydrophobicity in protein isolates from grain legumes, as seen here in MMGPI and 7EMGPI, which also display high surface hydrophobicity likely due to greater exposure of hydrophobic residues in the unfolded structure. These results align with previous findings for similar plant proteins. Aderinola et al. (2020) and Osemwota et al. (2021)) reported that unordered structures dominated the secondary structure of *Moringa oleifera* and lentil seed protein isolates, respectively. However, contrary to our findings, Illingworth et al. (2024) reported that α-helix and β-sheets predominated in quinoa protein isolates, accounting for about 58% of the structure, while D'Amico et al. (2017) also reported a predominance of β-sheets in amaranth protein isolates. This variability across different plant proteins may arise from their unique structural properties or extraction conditions.

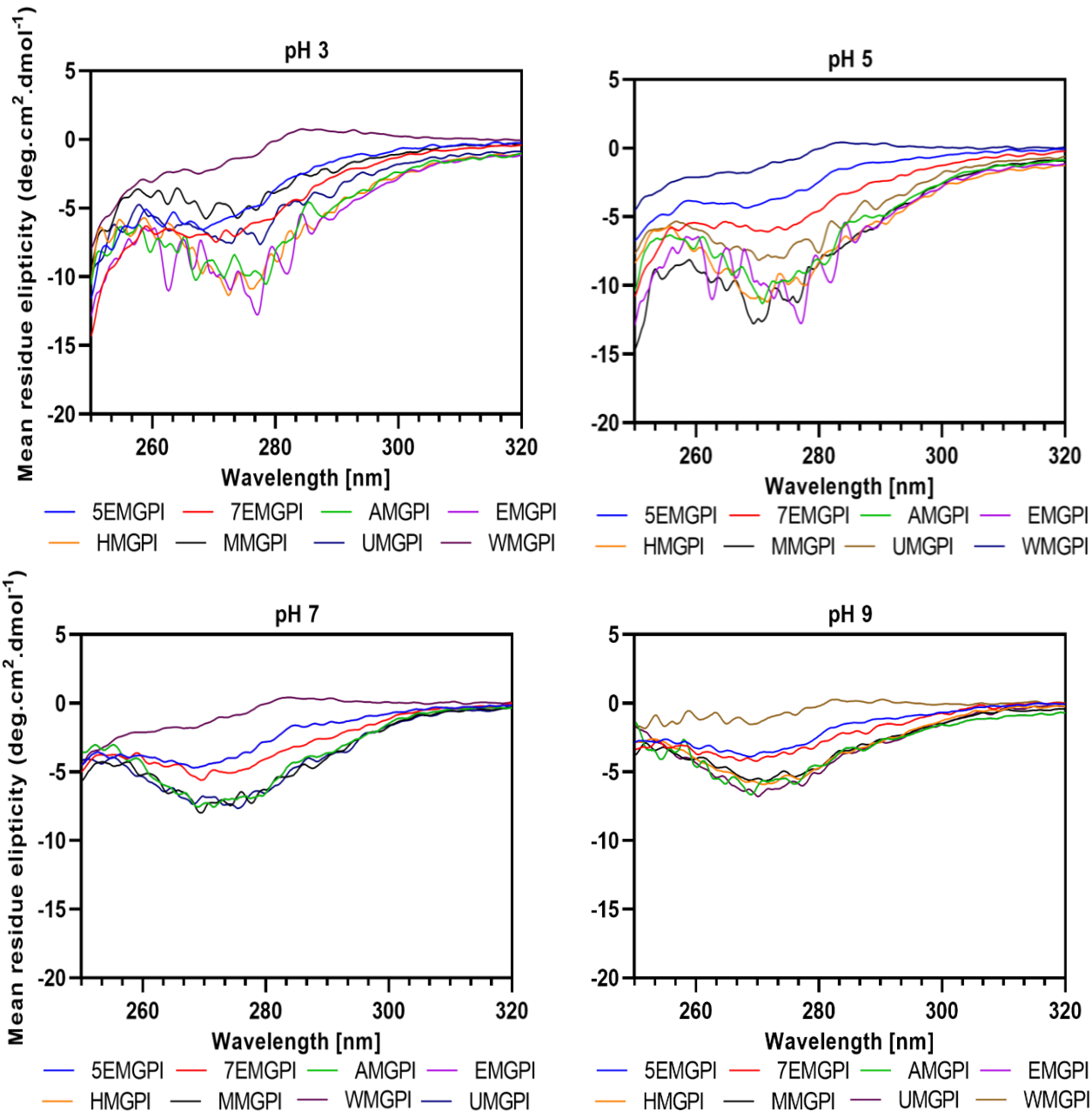


Figure 3. 5: Near UV of protein isolates obtained from undefatted MS meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI).

Table 3.6: Secondary structure fractions of protein isolates obtained from undefatted MS meal (UMGPI), or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI).

pH	Structure	5EMGPI	7EMGPI	AMGPI	EMGPI	HMGPI	MMGPI	UMGPI	WMGPI
3	α-helix	9.20±0.02	3.95±0.05	4.80±0.06	3.85±0.05	2.85±0.04	23.05±0.16	3.30±0.04	4.85±0.05
	β-sheet	26.40±0.10	35.60±0.16	32.15±0.14	35.59±0.16	41.65±0.19	7.90±0.05	39.45±0.18	33.55±0.15
	β-Turns	15.90±0.02	19.0±0.00	17.55±0.01	18.25±0.00	19.55±0.01	17.10±0.12	19.60±0.00	17.75±0.00
	Unordered	52.0±0.04	41.45±0.00	45.65±0.00	41.90±0.01	35.85±0.03	52.0±0.21	37.65±0.03	43.80±0.01
5	α-helix	5.05 ± 0.05	7.55 ± 0.02	7.80±0.04	5.60±0.05	6.20±0.05	3.75±0.05	4.45±0.06	4.65±0.05
	β-sheet	29.45±0.12	14.65±0.02	20.05±0.06	26.00±0.10	27.85±0.12	36.15±0.16	30.10±0.13	33.70±0.15
	β-Turns	16.65±0.01	13.75±0.03	14.50±0.02	16.10±0.02	16.80±0.02	18.55±0.00	16.90±0.01	17.95±0.00
	Unordered	48.90±0.02	64.15±0.15	57.65±0.08	52.30±0.05	49.15±0.06	41.55±0.02	48.55±0.02	43.7±0.02
7	α-helix	6.20±0.05	9.10±0.03	5.90±0.05	8.05±0.03	4.95±0.05	7.05±0.02	7.35±0.03	5.55±0.05
	β-sheet	23.85±0.09	10.65±0.01	22.00±0.07	18.10±0.05	29.95±0.13	13.35±0.01	14.85±0.02	26.20±0.10
	β-Turns	15.55±0.03	14.85±0.01	15.40±0.03	13.95±0.03	16.95±0.01	18.00±0.03	13.95±0.02	15.75±0.01
	Unordered	54.45±0.07	65.35±0.11	56.70±0.09	59.95±0.10	48.15±0.01	61.55±0.10	63.90±0.14	52.45±0.05

9	α-helix	4.40±0.05	8.50±0.03	4.05±0.05	8.45±0.04	5.25±0.04	8.00±0.04	5.10±0.05	5.80±0.05
	β-sheet	35.65±0.16	22.00±0.08	35.75±0.16	22.40±0.08	34.00±0.15	21.70±0.08	30.15±0.13	29.80±0.13
	β-Turns	18.70±0.01	14.95±0.02	18.85±0.00	14.65±0.02	18.50±0.01	14.45±0.03	17.15±0.01	16.45±0.01
	Unordered	41.20±0.03	54.55±0.08	41.35±0.00	54.55±0.07	42.20±0.02	55.85±0.08	4.60±0.02	47.95±0.01

The data represent the mean values of duplicate readings with their corresponding standard deviations. Different superscripts within the same row signify statistically significant differences among the samples ($p < 0.05$).

3.3.13 Protein solubility

Protein solubility is a critical factor influencing the functional, sensory, and nutritional qualities of food products (Day et al., 2022). It affects essential aspects such as incorporation ease, stability, texture, and shelf life in food formulations (Hosseini & Jafari, 2020). Consequently, understanding the solubility behavior of food proteins is vital for optimizing product performance. Figure 3.6A illustrates the solubility curve of MSPI across a pH range of 3, 5, 7, and 9. The curve exhibits a U-shaped pattern, indicating that solubility decreases around pH 4 to 5 before increasing again in both the acidic (pH 3) and basic (pH 8 to 9) regions. This decrease in solubility at pH 4 to 5 suggests proximity to the isoelectric point (pI) of pH 4.5, where proteins carry no net charge and tend to aggregate, leading to reduced solubility. As the pH deviates from the pI, proteins acquire a net positive charge in acidic conditions and a net negative charge in alkaline conditions (Mundi & Aluko, 2012). This increase in electrostatic repulsion among the protein molecules minimizes aggregation and enhances solubility. This finding aligns with results observed in kidney bean seed protein fractions, rice bran protein isolate, pea protein isolate, Chinese quince seed protein isolate, and walnut protein isolate (Mundi & Aluko, 2012; Hu et al., 2017; Deng et al., 2019; Abd Rahim et al., 2024; Othmeni et al., 2024).

Interestingly, some samples, particularly WMGPI and EMGPI, exhibited a secondary dip in solubility at pH 7, which was unexpected given the distance from the pI. This behavior may be attributed to secondary aggregation caused by the exposure of hydrophobic regions, leading to clumping (Rajan et al., 2021). Previous research has shown that protein aggregates can form at neutral pH due to hydrophobic interactions, even if the isoelectric point is at a different pH (Ventri et al., 2007). Additionally, prior studies on plant-based proteins indicate that processing techniques, including the choice of defatting solvents, can significantly impact protein

conformation and solubility behavior (Akharume et al., 2021; Kamal et al., 2021). Structural modifications, such as partial unfolding or aggregation, can expose hydrophobic regions or reduce the availability of charged residues, thereby shifting isoelectric behavior and extending the pH range of reduced solubility (Yang et al., 2024). Another factor contributing to the reduced solubility at pH 7 could be the interaction between polyphenols and proteins. Although polyphenols are not present in high quantities, they may form complexes with the proteins at pH 7 through hydrogen bonding or hydrophobic interactions. These complexes could lead to protein aggregation or precipitation, thereby reducing solubility. The highest solubility observed at pH 3 indicates that the MS proteins possess a strong positive charge at this acidic pH, minimizing aggregation and maintaining good dispersion in solution. This finding aligns with studies on kidney bean albumin and buckwheat protein isolate, which also exhibited higher solubility at pH 3. However, some pulse proteins have shown increased solubility at alkaline pH levels (Ladjal-Ettoumi et al., 2016; Rahmati et al., 2018; Osemwota et al., 2021). The observed solubility of about 30% at pH 9 in this study is comparable to that of pea protein isolates (Shand et al., 2007; Adebiyi & Aluko, 2011).

Among the MSPI samples, UMGPI exhibited the highest solubility across all pH levels, particularly at pH 3. This is likely due to the retention of the proteins' natural conformation, which prevents excessive unfolding or aggregation that can occur during defatting (Levental & Lyman, 2023). The preservation of structural integrity allows protein isolated from the undefatted meal (UMGPI) to maintain higher solubility over a broader pH range when compared to proteins isolated from the defatted meals. In contrast, 5EMGPI showed the lowest solubility across all pH levels except at pH 7, where WMGPI had the lowest solubility. The reduced solubility of WMGPI may result from a higher retention of hydrophobic compounds. Conversely, AMGPI demonstrated

relatively higher solubility at most pH levels except pH 9, which suggests that acetone effectively removes non-polar compounds like lipids and free fatty acids without significantly denaturing proteins (Abhari & Khaneghah, 2020). This selective removal may enhance the exposure of hydrophilic groups on the protein surface, promoting interactions with water and improving solubility.

3.3.14 Heat coagulability

Figure 3.6B displays the heat coagulability (%) of MSPI across a pH range of 3 to 9. Heat coagulability reflects the degree of protein aggregation upon heating, influenced by pH, protein structure, and pre-treatment methods. At pH 3, all samples show minimal coagulability (Wu et al., 2024). However, there is a sharp increase between pH 4 and 5, with a peak near neutral pH (6 – 7). At pH 8 – 9, coagulability slightly decreases, indicating reduced aggregation under alkaline conditions. This outcome contrasts with the findings of (Osemwota et al., 2021) for lentil protein isolate, which reported a more pronounced decline in heat susceptibility at alkaline pH. WMGPI consistently exhibited the highest coagulability across all pH levels, reaching a peak of 90% at pH 7, indicating rapid aggregation upon heating. In comparison, both HMGPI and UMGPI show lower coagulability across the pH range, reflecting weaker heat-induced aggregation. The heat coagulability curve inversely correlates with the MSPI solubility curve. This agrees with previous research reports, which show that reduced solubility during heating is associated with protein aggregation caused by molecules unfolding (Chen et al., 2018). Similarly, Grossmann and McClements (2023) confirmed that lower solubility promotes heat-induced aggregation.

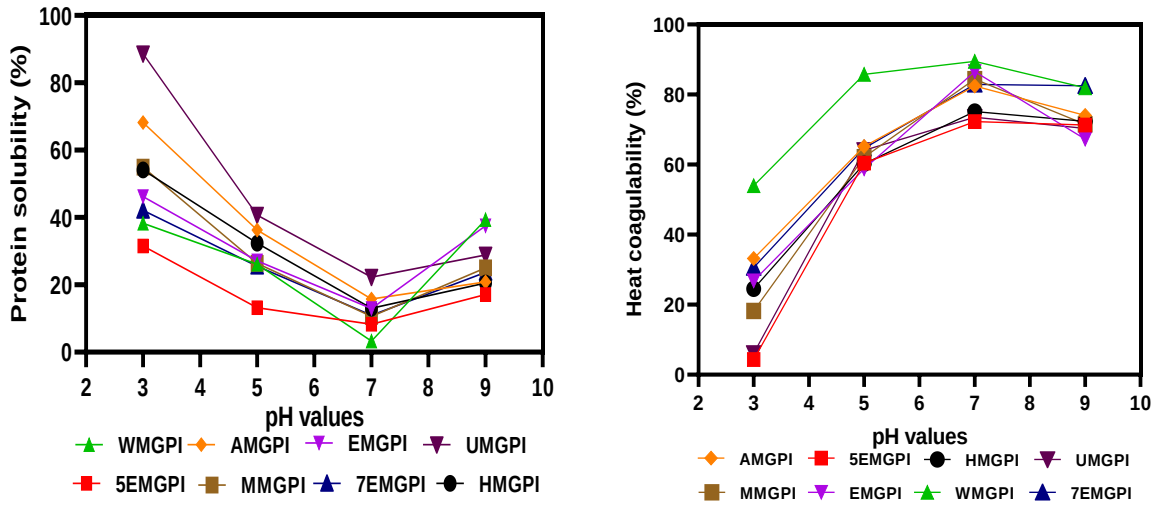


Figure 3. 6: Protein solubility (A) and heat coagulability (B) of protein isolates obtained from undefatted *Moringa Stenopeta*la meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI).

3.3.15 Emulsion formation & stability

Figure 3.7 presents the oil droplet size ($d_{3,2)}$ of emulsion formed with the MSPI at different protein concentrations (10, 15, and 20 mg/mL) and pH values (3, 5, 7, and 9). During emulsification, proteins interacts with the oil-water interface, where they adsorb and create viscoelastic films that surround the oil droplets (Zhou et al., 2021). In this structure, hydrophobic groups face the oil phase, while hydrophilic groups orient towards the aqueous phase (Zhou et al., 2021). Our findings highlight that the emulsifying properties of MSPI are strongly pH-dependent, aligning with prior studies showing that emulsion capacity is influenced by pH, which affects the hydrophilic-hydrophobic balance (Illingworth et al., 2024). At pH 7 and 9, larger oil droplets were observed, indicating reduced emulsifying efficiency under alkaline conditions. In contrast, smaller droplets were formed at pH 3 and 5, suggesting better emulsifying performance in acidic environments. These results align with the findings from Osemwota et al. (2021), who reported similar trends for lentil protein isolates prepared using NaCl membrane methods. The effect of protein concentration was minimal at pH 3 and 5 but more pronounced at pH 7 and 9, where higher concentrations resulted in larger droplets, which suggest that increased electrostatic repulsions between the proteins prevent adequate formation of interfacial membranes needed for emulsification of the oil droplets. The performance of MSPI varies by type. UMGPI, WMGPI, and 5EMGPI consistently formed smaller droplets across all pH levels, including pH 7 and 9, indicating superior emulsifying properties. Conversely, EMGPI, 7EMGPI, and MMGPI produced larger droplets, particularly at pH 9. This suggests that, despite the high hydrophobic amino acid content in 7EMGPI and MMGPI, which enhances emulsifying properties by reducing surface tension (Zhang et al., 2022), the use of methanol and high concentrations of ethanol during

defatting may have induced structural changes in the proteins, thereby reducing their emulsion forming ability

These findings have practical relevance for emulsified food products such as sauces, dressings, and nutraceutical beverages. The pH-dependent emulsifying behavior suggests that these isolates may perform better in acidic systems like yogurt, fruit beverages, and acidic sauces, where smaller droplet sizes and enhanced emulsifying properties are preferred. The oil droplet sizes reported here are smaller (indicating better emulsions) than the 18–85 µm reported for canola protein isolates (Tan et al., 2014) and 22–100 µm for pea protein isolates (Chao & Aluko, 2018) but higher than 18–30 µm for kidney bean protein isolates (Mundi & Aluko, 2012) at pH 3 – 9. These differences underscore the importance of raw material selection in determining protein functionality.

As shown in Figure 3.8, emulsion stability was assessed 30 min after emulsion formation and under varying protein concentrations (10, 15, and 20 mg/mL) and pH (3, 5, 7, and 9). Emulsion stability reflects the ability of protein-stabilized emulsions to resist phase separation over time (Fan et al., 2022). Stability was lowest at pH 3 and highest at pH 9. At pH 7 and 9, emulsions exhibited superior stability compared to those at pH 3 and 5, consistent with findings for hemp seed protein isolate, where stability decreased under acidic conditions (Malomo & Aluko. 2015). Increased emulsion stability at higher pH is attributed to enhanced surface charge, which contributes to increased repulsions between the interfacial protein films and hence reduced coalescence of oil droplets (Fan et al., 2022). This supports the formation of stable emulsions despite the larger droplet sizes observed at pH 7 and 9. Although smaller droplet sizes typically improve stability by increasing the surface area and reducing coalescence, our results show the opposite. Larger droplets at higher pH levels exhibited greater stability, which aligns with the

report of Kaushik et al. (2016), who noted that droplet size alone does not determine emulsion stability. At pH 3, despite high solubility, the acidic environment likely induced structural changes in the protein that destabilized the emulsion. Acid-induced conformational changes may weaken interfacial protein film, increasing susceptibility to coalescence and phase separation (Han et al., 2021). Similarly, at pH 5 close to the pI of MSPI, emulsion stability was reduced. Near the pI, solubility decreases, and proteins carry minimal net charge, leading to reduced electrostatic repulsion and weak interfacial films, which promote droplet coalescence and destabilization (Albano et al., 2019). These results are consistent with findings from a previous study on sesame seed protein isolates, where emulsion stability declined near the pI (Rezaei et al., 2022). Among the tested concentrations, emulsions with 20 mg/mL protein consistently showed the highest stability across most pH levels. Higher protein concentrations likely improved stability by increasing the availability of protein molecules at the interface, which enabled formation of thick films that are resistant to fast coalescence and phase separation (Albano et al., 2019). WMGPI exhibited excellent stability at pH 5, suggesting that defatting the MS meal with water produced proteins with better ability of form strong and cohesive interfacial membranes near the pI. HMGPI and AMGPI also showed higher stability, particularly at pH 9, highlighting the positive effect of these solvents on protein structure with respect to emulsion stability. These results align with previous report showing that hexane-defatted insect protein isolate achieved the highest emulsion stability (Kim et al., 2021). It is important to note that emulsion stability depends not only on solubility but also on other factors, such as phase-to-volume ratio, droplet size distribution, interfacial properties, viscosity, and density differences between phases (Mohammed et al., 2018). Each parameter plays a crucial role in determining the overall stability of protein-stabilized emulsions.

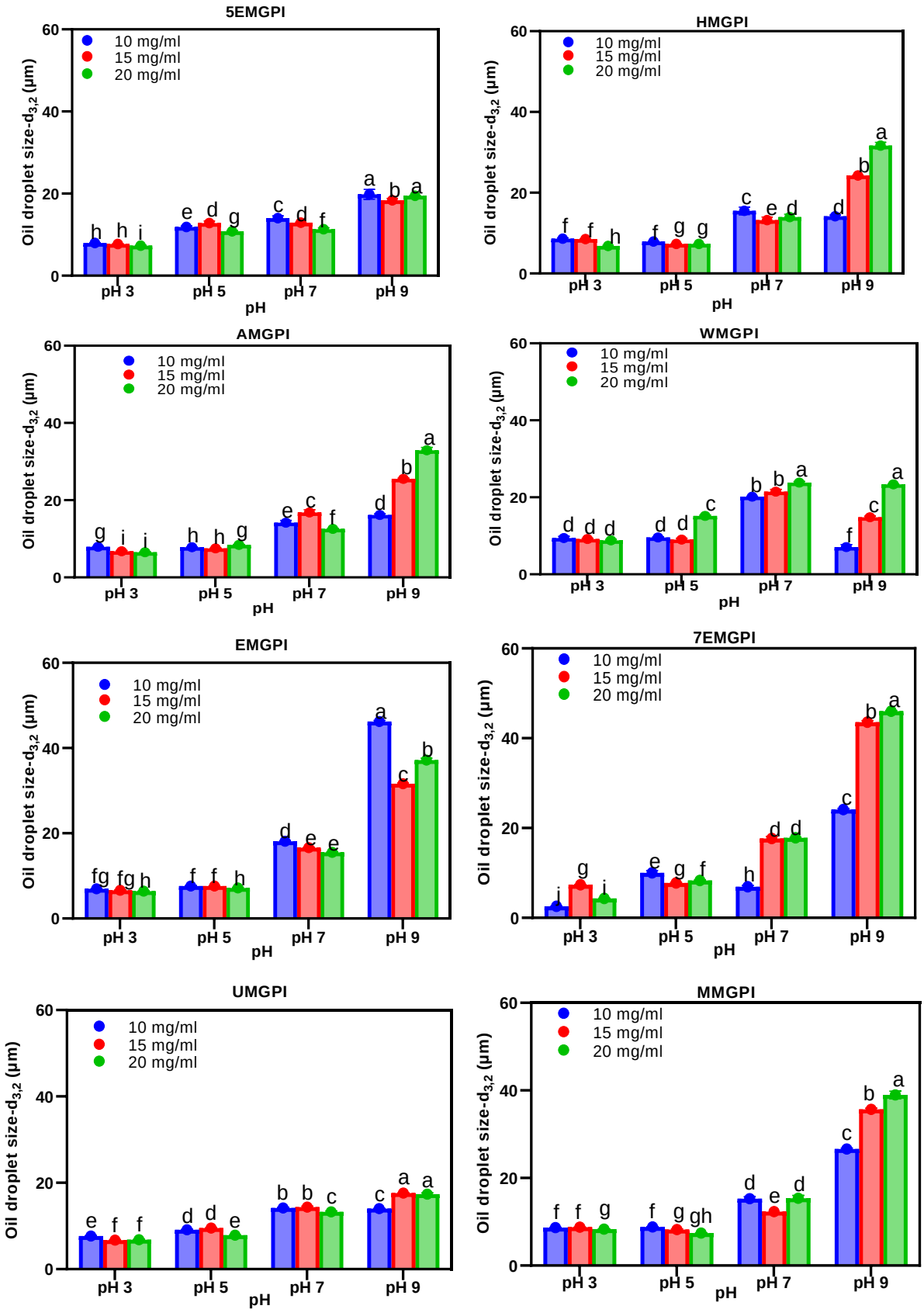
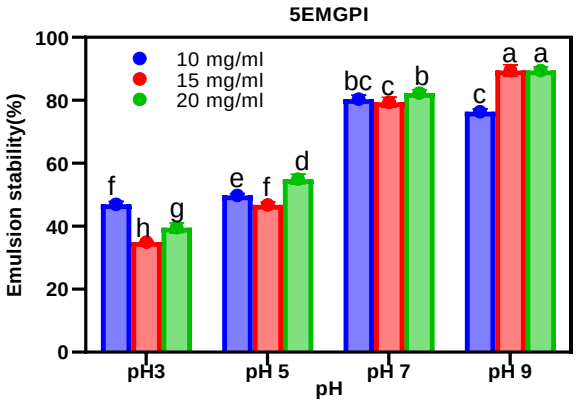
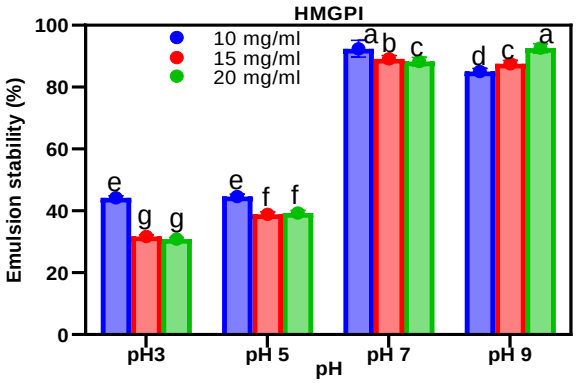
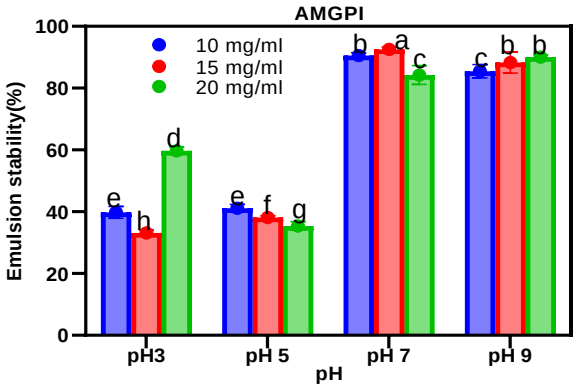
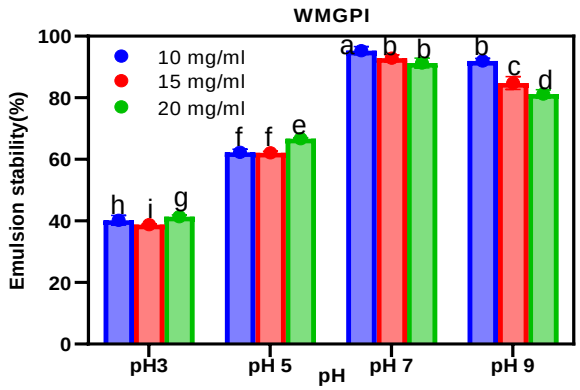

5EMGPI
HMGPI
AMGPI
WMGPI
EMGPI
7EMGPI
UMGPI
MMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Oil droplet size-d3,2 (µm)
pH
pH 3
pH 5
pH 7
pH 9
0
20
40
60

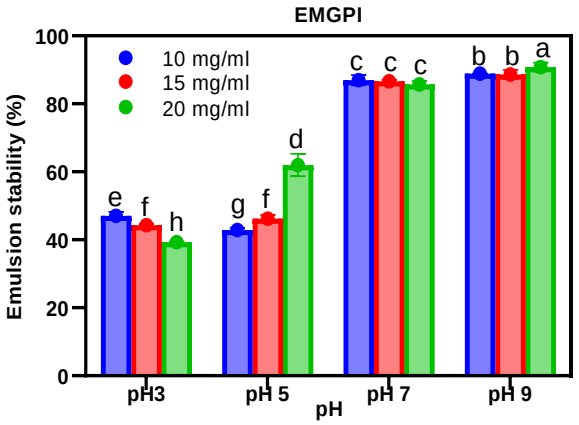
Figure 3. 7: Oil droplet size of emulsions formed by protein isolates obtained from undefatted MS meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI).

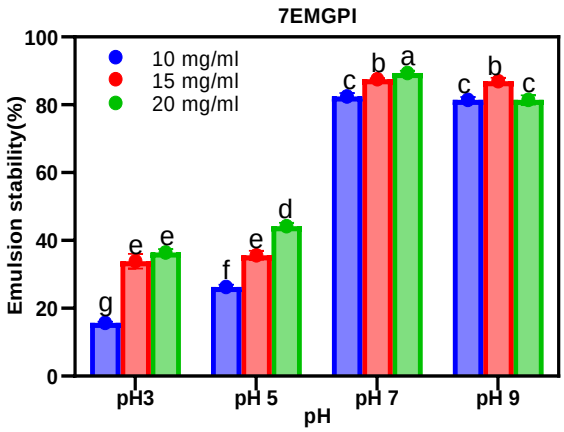

5EMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Emulsion stability(%)
pH3
pH 5
pH 7
pH 9
pH

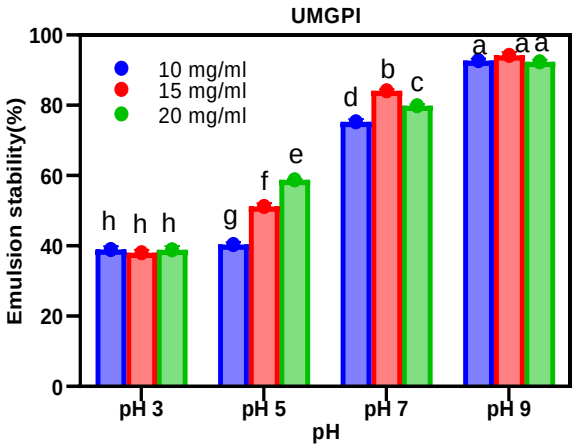

HMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Emulsion stability (%)
pH3
pH 5
pH 7
pH 9
pH


AMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Emulsion stability(%)
pH3
pH 5
pH 7
pH 9
pH


WMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Emulsion stability(%)
pH3
pH 5
pH 7
pH 9
pH


EMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Emulsion stability (%)
pH3
pH 5
pH 7
pH 9
pH


7EMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Emulsion stability(%)
pH3
pH 5
pH 7
pH 9
pH


UMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Emulsion stability(%)
pH 3
pH 5
pH 7
pH 9
pH

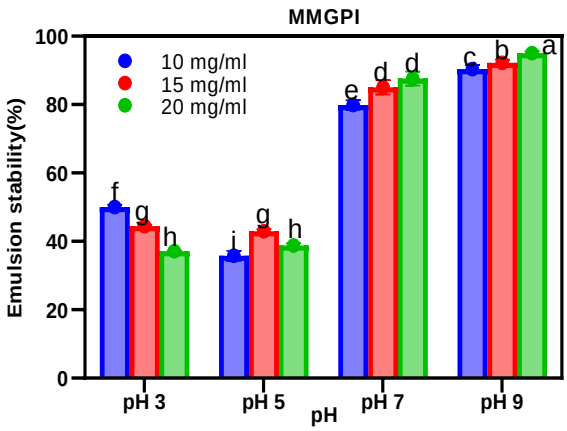

MMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Emulsion stability(%)
pH 3
pH 5
pH 7
pH 9
pH

Figure 3. 8: Stability of emulsions formed with protein isolates obtained from undefatted MS meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI).

3.3.16 Foaming capacity and foam stability

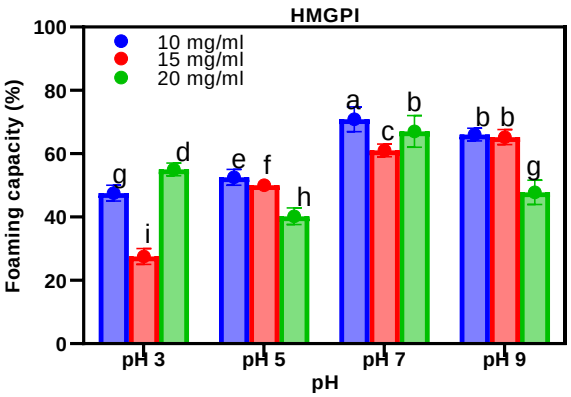
Figure 3.9 presents the foaming capacity (FC) of MSPI at varying pH values (3, 5, 7, and 9) and concentrations (10, 15, and 20 mg/mL). FC refers to a protein's ability to generate foam when air is incorporated into a liquid, an essential property for food formulations because foam contributes to texture, appearance, and sensory appeal (Ellis & Lazidis, 2018). Several factors influence FC, including adsorption at the air-water interface, protein solubility, conformational changes, intermolecular interactions, and the formation of cohesive, viscoelastic films (Mundi & Aluko, 2012). The results indicate that FC is highly pH-dependent, with higher values (~ 80 %) generally observed at pH 7 and 9 when compared to pH 3 and 5. This aligns with Chao and Aluko (2018), who reported that FC tends to be higher at neutral or alkaline pH levels due to increased solubility, which enhances protein flexibility and better ability to encapsulate air bubbles. The variation in FC across concentrations was also notable. In some conditions, FC increased with higher concentrations, while in others, it decreased, indicating that concentration plays a nuanced role in foam formation.

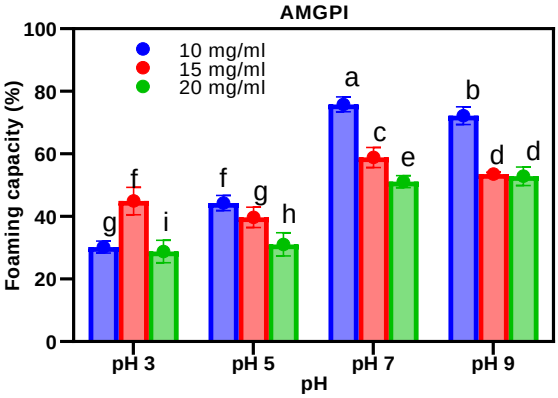
The low foam capacity (FC) at pH 5 can be attributed to the decreased solubility of MSPI near the protein's isoelectric point of pH 4.5. At this pH, the protein molecules have a minimal net charge, which encourages aggregation and precipitation. This reduces the protein's diffusion at the air-water interface and limits its ability to trap air bubbles (Othmeni et al., 2024). Similar behavior has been reported for egg yolk hydrolysates, where low solubility coincided with poor FC (Bao et al., 2017). Interestingly, despite the highest protein solubility being observed at pH 3, the FC at this pH was lower than expected. This could be due to structural changes induced by the acidic environment, such as denaturation or alterations in surface hydrophobicity, which impair the

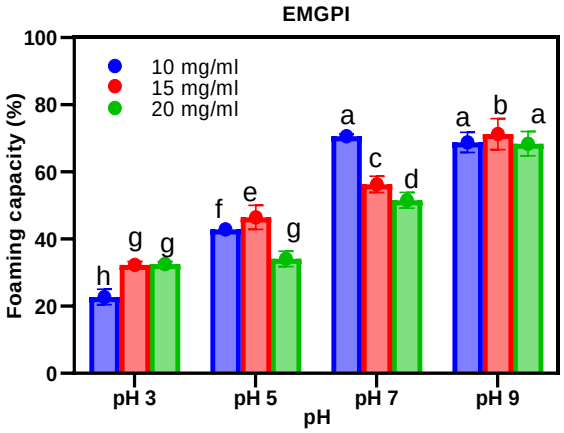
proteins' ability to unfold and stabilize air bubbles (Srivastava & Alam, 2020). These findings emphasize that high solubility alone does not guarantee improved foaming properties.

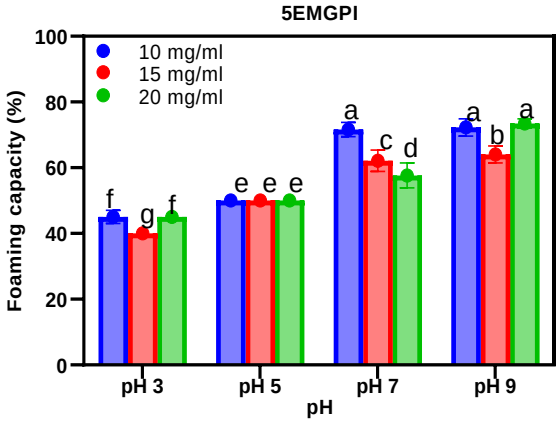
The increased foam capacity (FC) at pH 7, despite some aggregation, supports the idea that partial unfolding and aggregation can enhance foam formation by improving interfacial properties. This observation aligns with El Nasri and Tinay (2007), who reported higher FC due to increased protein aggregation. Similarly, Benelhadj et al. (2016) demonstrated that raising pH enhanced the FC of algae protein isolates, suggesting that factors beyond solubility contribute to foam formation. At pH 9, the FC was notably high, likely due to improved protein solubility and conformational flexibility in alkaline conditions. This observation is further supported by the increased unordered structure at pH 7 and 9 for most samples, as revealed by secondary structure analysis. Zhan et al. (2020) also found that proteins with flexible, random coil structures were more effective in rearranging at the interface during emulsions and foam formation than highly ordered proteins, which do not unfold as easily to interact with the surrounding medium. Together, these findings suggest that enhanced conformational flexibility and protein structure at higher pH levels may play a crucial role in promoting foam stability and formation, beyond the effects of solubility alone. Among the protein isolates tested, UMGPI showed the highest FC at pH 7 and 9. Solvent type also affected the foaming properties: 7EMGPI displayed superior FC under these conditions, indicating its potential for improving foaming performance in food systems. In contrast, WMGPI had the lowest FC at both pH 7 and 9. However, at pH 3 and 5, both HMGPI and WMGPI exhibited higher FC, suggesting specific effects of solvent defatting on protein conformation. These findings highlight the potential applications of MSPI in food products that require strong foaming properties, such as whipped toppings and foamy beverages.

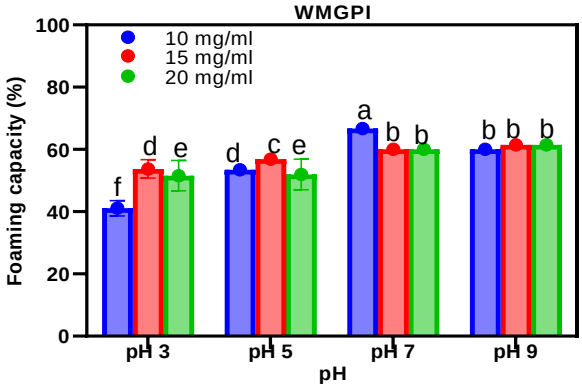
Figure 3.10 also presents foam stability (FS) results for MSPI across different pH levels and concentrations. FS reflects the persistence of foam over time, a crucial property in food formulations that rely on stable air-water interface. As with FC, FS was influenced by pH, concentration, and solvent type. The results show improved FS at pH 3, with the lowest stability observed at pH 5. This agrees with findings from previous research, where pea protein concentrates exhibited better FS at pH 3 than at pH 5 (Asen & Aluko, 2022). Similarly, Barac et al. (2015) reported that both native and denatured legume proteins displayed enhanced FS under neutral and alkaline conditions. In this study, FS increased at pH 7 and 9, mirroring the trend observed for FC. This suggests that MSPI performs optimally in both foam formation and stability at neutral to alkaline pH levels, making it suitable for applications in whipped toppings, beverages, and desserts. The increased FS at pH 7 and 9 could be attributed to higher levels of electrostatic charges that reduced interactions between the foam particles to prevent foam collapse. Notably, 5EMGPI and WMGPI exhibited the lowest FS at pH 3 and 5, while EMGPI showed poor FS at pH 7 and 9. These differences highlight the importance of the defatting solvent on protein functionality. The interactions between protein structure, solubility, concentration, and defatting solvent play critical roles in determining both FC and FS. These findings demonstrate that MSPI has strong potential as a functional ingredient in food systems that require foaming properties. Understanding the interplay between pH, concentration, and defatting methods can help optimize MSPI's performance in various food formulations.

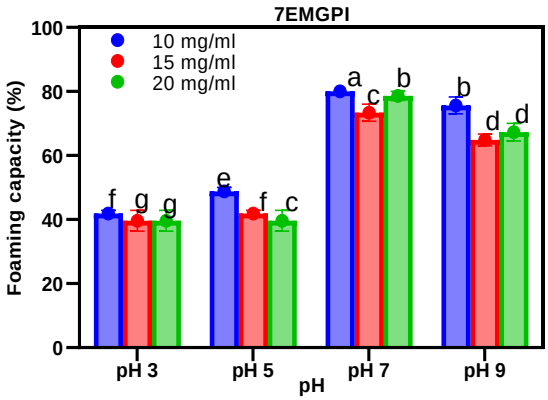

HMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Foaming capacity (%)
pH 3
pH 5
pH 7
pH 9
pH

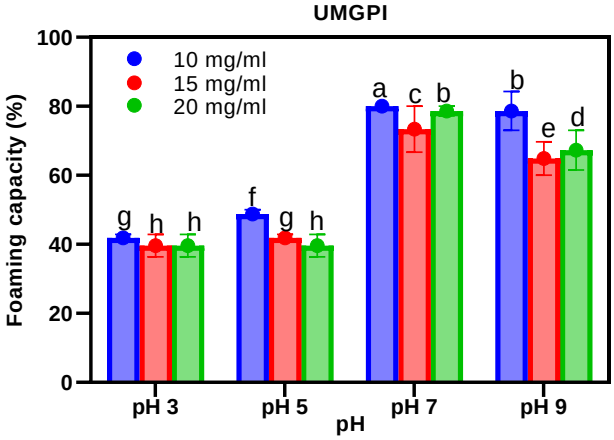

AMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Foaming capacity (%)
pH 3
pH 5
pH 7
pH 9
pH


EMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Foaming capacity (%)
pH 3
pH 5
pH 7
pH 9
pH


5EMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Foaming capacity (%)
pH 3
pH 5
pH 7
pH 9
pH


WMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Foaming capacity (%)
pH 3
pH 5
pH 7
pH 9
pH


7EMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Foaming capacity (%)
pH 3
pH 5
pH 7
pH 9
pH


UMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Foaming capacity (%)
pH 3
pH 5
pH 7
pH 9
pH

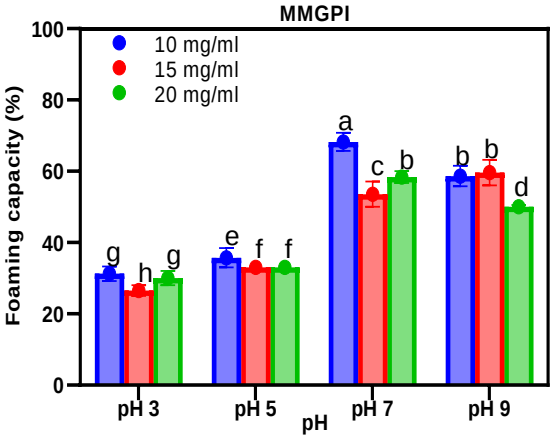
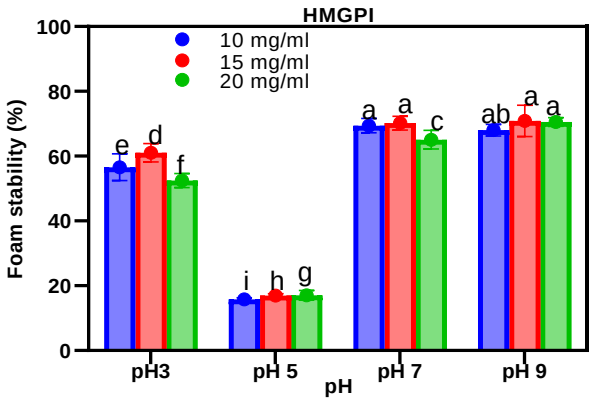
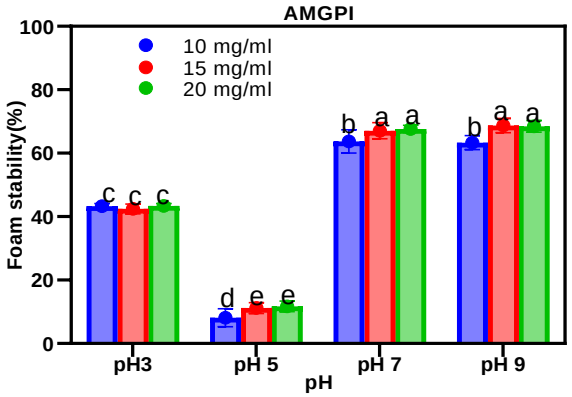
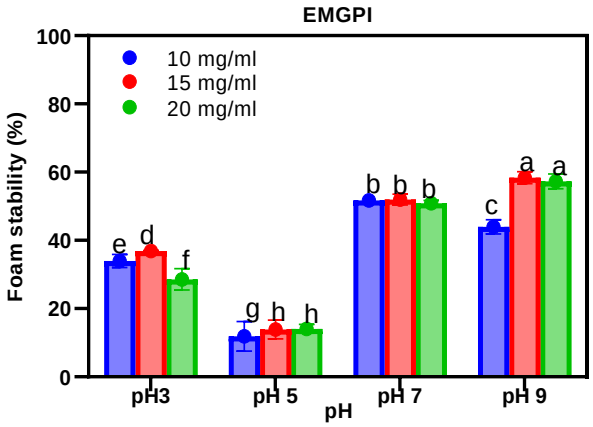
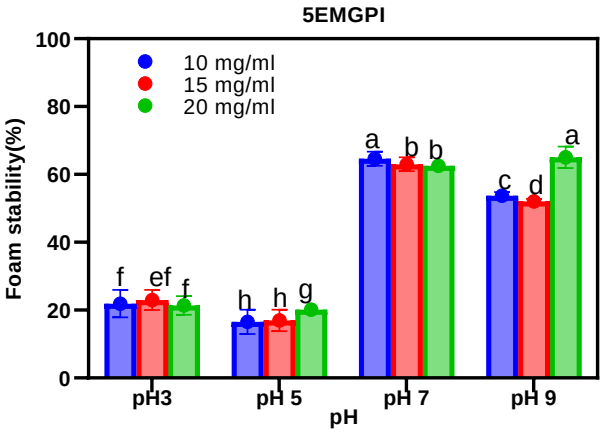

MMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Foaming capacity (%)
pH 3
pH 5
pH 7
pH 9
pH

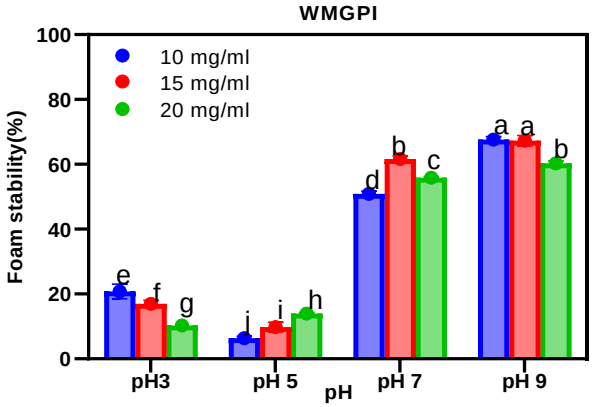
Figure 3. 9: Foaming capacity of protein isolates obtained from undefatted *Moringa stenopetala* meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI).

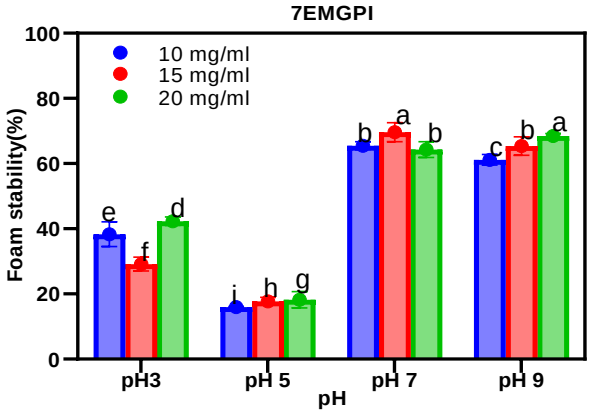

HMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Foam stability (%)
pH3
pH 5
pH 7
pH 9
pH

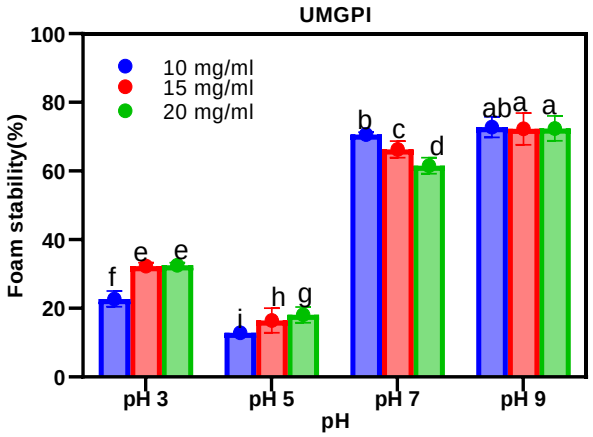

AMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Foam stability(%)
pH3
pH 5
pH 7
pH 9
pH


EMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Foam stability (%)
pH3
pH 5
pH 7
pH 9
pH


5EMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Foam stability(%)
pH3
pH 5
pH 7
pH 9
pH


WMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Foam stability(%)
pH3
pH 5
pH 7
pH 9
pH


7EMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Foam stability(%)
pH3
pH 5
pH 7
pH 9
pH


UMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Foam stability(%)
pH 3
pH 5
pH 7
pH 9
pH

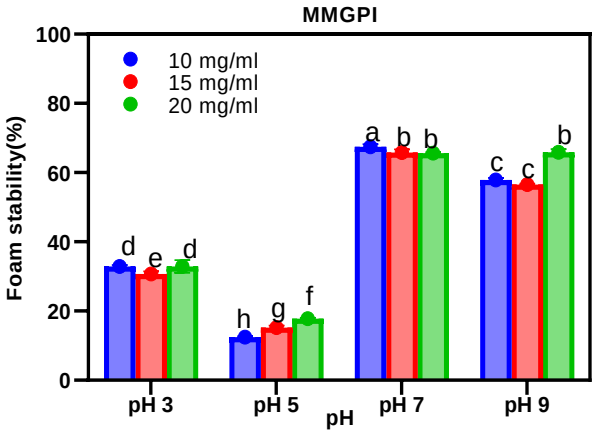

MMGPI
10 mg/ml
15 mg/ml
20 mg/ml
Foam stability(%)
pH 3
pH 5
pH 7
pH 9
pH

Figure 3. 10: Stability of foams produced by protein isolates obtained from undefatted *Moringa stenopetala* meal (UMGPI) or meal defatted with; acetone (AMGPI), 100% ethanol (EMGPI), hexane (HMGPI), methanol (MMGPI), 70% ethanol (7EMGPI), 50% ethanol (5EMGPI), and water (WMGPI).

3.4 Conclusion

This study examined how the type of defatting solvent affects the physicochemical and functional properties of MSPI obtained from the defatted MS meal. The results indicate that pH 3 provided an environment for the highest solubility, making it suitable for applications that require good dispersion, such as protein-enriched beverages. However, the acidic nature of pH 3 may limit its applicability in certain formulations such as emulsions. On the other hand, pH 7 and pH 9 are more appropriate for emulsions and foams, as these conditions promote stability and air incorporation. The results also show that the UMGPI retained superior functionality across multiple parameters, suggesting that lack of defatting may have preserved the native protein structure during processing, which enhanced its overall performance. Among the protein samples, MMGPI from methanol-defatted meal exhibited a more balanced amino acid profile, comparable to that of 7EMGPI from 70% ethanol-defatted meal. Additionally, MMGPI displayed a lower bitterness intensity, suggesting that methanol defatting may be more effective in reducing the bitter taste compared other defatting solvents tested. AMGPI from acetone-defatted meal is the most balanced with respect to protein structural integrity and functionality across different tests. AMGPI demonstrated high solubility, good emulsifying properties, reduced heat coagulability, and strong foaming performance across various pH levels. When comparing different ethanol concentrations for MS meal defatting, 70% ethanol was the most effective in enhancing protein functionality of the extracted proteins. 7EMGPI offers a well-rounded combination of high solubility, and stable emulsifying properties. This solvent may have contributed to maintaining a structure that enhanced protein functionality better than both 5EMGPI and EMGPI samples, making the 7EMGPI an optimal choice for food applications. These findings emphasize the importance of selecting the

appropriate defatting solvents to enhance the performance of MSPI in various food and nutraceutical formulations.

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TRANSITION STATEMENT

Understanding the physicochemical and functional properties of MSPI lays a critical foundation for further investigation into the bioactivity of these proteins, particularly concerning their enzyme inhibition potential. Given that MSPI demonstrates enhanced solubility, emulsification, and foaming capabilities, it is essential to explore how these functional attributes relate to the biological activity of the peptides derived from them. Enzyme inhibition is a vital aspect of many health-promoting properties, as bioactive peptides can play significant roles in regulating various physiological processes. Therefore, delving deeper into the enzyme inhibition potentials of bioactive peptides obtained from MS seeds not only complements the knowledge gained from studying MSPI's functional properties but also opens new avenues for developing nutraceuticals that leverage these bioactive compounds for health benefits.

CHAPTER FOUR

MANUSCRIPT TWO

4.0 IN VITRO BIOACTIVE PROPERTIES OF *MORINGA STENOPETALA* SEED ENZYMATIC PROTEIN HYDROLYSATES

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4.1 Introduction

Plants have long been recognized as vital sources of traditional medicine, providing healthcare solutions for millions worldwide (Sen & Chakraborty, 2015). They also serve as valuable resources for modern drug development, meeting the increasing demand for new medicines as the global population grows (Astutik & Pretzsch, 2019). Today, over 3.5 billion people rely on plants to treat both human and livestock diseases (Gobana et al., 2023). Choudhary et al. (2021) noted that plants contain a wide variety of bioactive compounds, present in various parts, including seeds, which are effective against infectious diseases. These natural remedies often come with fewer, or no side effects compared to synthetic drugs, making them an attractive alternative (Anand et al., 2019).

Moringa stenopetala (MS), a plant in the Moringaceae family, has been widely utilized in ancient healing practices for centuries (Hamza & Azmachi, 2017). The genus Moringa, consisting of about 14 species, includes MS, which is primarily found in southern Ethiopia at elevations of 1100–1600 meters (Chekesa & Mekonnen, 2015; Mikore & Mulugeta, 2017). The healing powers of MS are largely ascribed to its rich profile of phytochemicals and other plant metabolites. However, little attention has been given to the bioactive peptides derived from its seeds, which may significantly contribute to its therapeutic effects. Bioactive peptides are particularly promising for managing chronic diseases, such as hypertension (Singh et al., 2022), though this area remains underexplored for MS.

Bioactive peptides, generally inactive within the primary structure of proteins, are released through enzymatic hydrolysis (Aluko, 2015). These short protein fragments have been widely studied for their therapeutic effects. Upon hydrolysis, MS proteins release bioactive peptides that exhibit various health-promoting properties, such as antihypertensive, antioxidant, and anti-

diabetic activities (Majumder & Wu, 2015; Olusola et al., 2018; Famuwagun et al., 2020). For instance, Aderinola et al. (2018) reported that proteolytic breakdown of plant seed proteins with the use of alcalase, trypsin, and pepsin enzyme can generate bioactive peptides from moringa seeds, showing promise as ingredients for nutraceutical and functional food formulation.

Another body of research demonstrates that food-derived peptides act as potent antihypertensive agents by inhibiting enzymes involved in the regulation of blood pressure, such as the angiotensin-converting enzyme (ACE) and renin (Sánchez-Rivera et al., 2016; He et al., 2013). Additionally, these peptides exhibit strong antioxidant effects, neutralizing harmful free radicals that contribute to oxidative stress (Girgih et al., 2015). Protein hydrolysates from watermelon seeds have shown α-amylase inhibition (Famuwagun et al., 2021), while peptides from pea seeds and potato tubers have demonstrated inhibitory effects on both α-glucosidase and α-amylase (Awosika & Aluko, 2019; Marthandam Asokan et al., 2019).

Given these findings, this growing compilation of findings highlights the multifunctional potential of food-derived peptides as bioactive compounds against chronic diseases like hypertension, diabetes, obesity, and oxidative stress. As research expands, these peptides may offer safer, natural alternatives to synthetic drugs, providing multiple health benefits in nutraceutical and functional food development. Despite extensive studies on bioactive peptides from other plant proteins, *Moringa stenopetala* seed protein hydrolysates (MSPH) remain largely unexplored. This research aims to address this knowledge gap by investigating MSPH's enzyme-inhibitory potential, particularly against metabolic enzymes like α-amylase and α-glucosidase, as well as its antioxidant and antihypertensive activities. By examining these properties, this research contributes to the innovation of foods with functional properties and nutraceutical benefits targeting metabolic disorders, offering accessible, natural approaches to health management

through MS bioactive peptides. This chapter will also explore the impact of different hydrolyzing enzymes on MS peptide functionality.

4.2 Materials and Methods

4.2.1 Raw materials and sample preparation

MS seed meal was obtained through mechanical oil pressing from BioTEI Inc. (Winnipeg, MB, Canada) and stored at -20°C until use. The meal was ground using a coffee grinder (PG-13658FA-CAN) and mixed with 1 M NaOH at a 1:5 (w/v) ratio. The mixture was stirred for 1 hour and then centrifuged at 8000 × g for 1 hour at room temperature. The supernatant was adjusted to pH 4.5 using 2.5 M HCl and refrigerated for 12 hours. Subsequently, the acidified mixture was centrifuged again at 8000 × g for 1 hour at 4°C. The resulting precipitate was freeze-dried and stored at -20°C as MS protein isolate (MSPI). Alcalase, flavourzyme, pepsin, pancreatin and all analytical grade chemicals used were sourced from Sigma Aldrich (St. Louis, MO, USA) or Fisher Scientific (Oakville, ON, Canada).

4.2.2 Protein hydrolysis

The hydrolysis of MSPI was carried out using three distinct enzymes under specific conditions (Figure 4.1), following the protocol of He et al. (2013) with minor adjustments. Alcalase hydrolysis was performed at 50°C and pH 8.0 for 4 h, flavourzyme at 50°C and pH 6.5 for 4 h, and a two-step process was applied for pepsin and pancreatin: pepsin hydrolysis was conducted at 37°C and pH 2.0 for 2 h, followed by pancreatin hydrolysis at 37°C and pH 7.5 for an additional 2 h. A 5% (w/v) suspension of MSPI, based on protein content, was prepared by dispersing the isolate in deionized water in a glass beaker equipped with a mechanical stirrer. The mixture was heated to the target temperature, and the pH was adjusted using 1 M NaOH or 1 M

HCl before enzyme addition. Proteases were added at an enzyme-to-substrate (E/S) ratio of 4:100 for all three processes. Temperature and pH were controlled throughout hydrolysis to maintain optimal enzyme activity. After hydrolysis, enzyme inactivation was achieved by adjusting the mixture to pH 4.5 with 2 M HCl, followed by placing the reaction vessel in a boiling water bath (95°C) for 15 min. The hydrolysates were separated from undigested protein fractions through centrifugation at 8000 × g for 30 min at 4°C. The supernatant containing peptides was collected, freeze-dried, and stored at -20°C as MS protein hydrolysates (MSPH) for further studies. The protein content of the freeze-dried MSPH was determined using the modified Lowry method as outlined by Markwell et al. (1978).

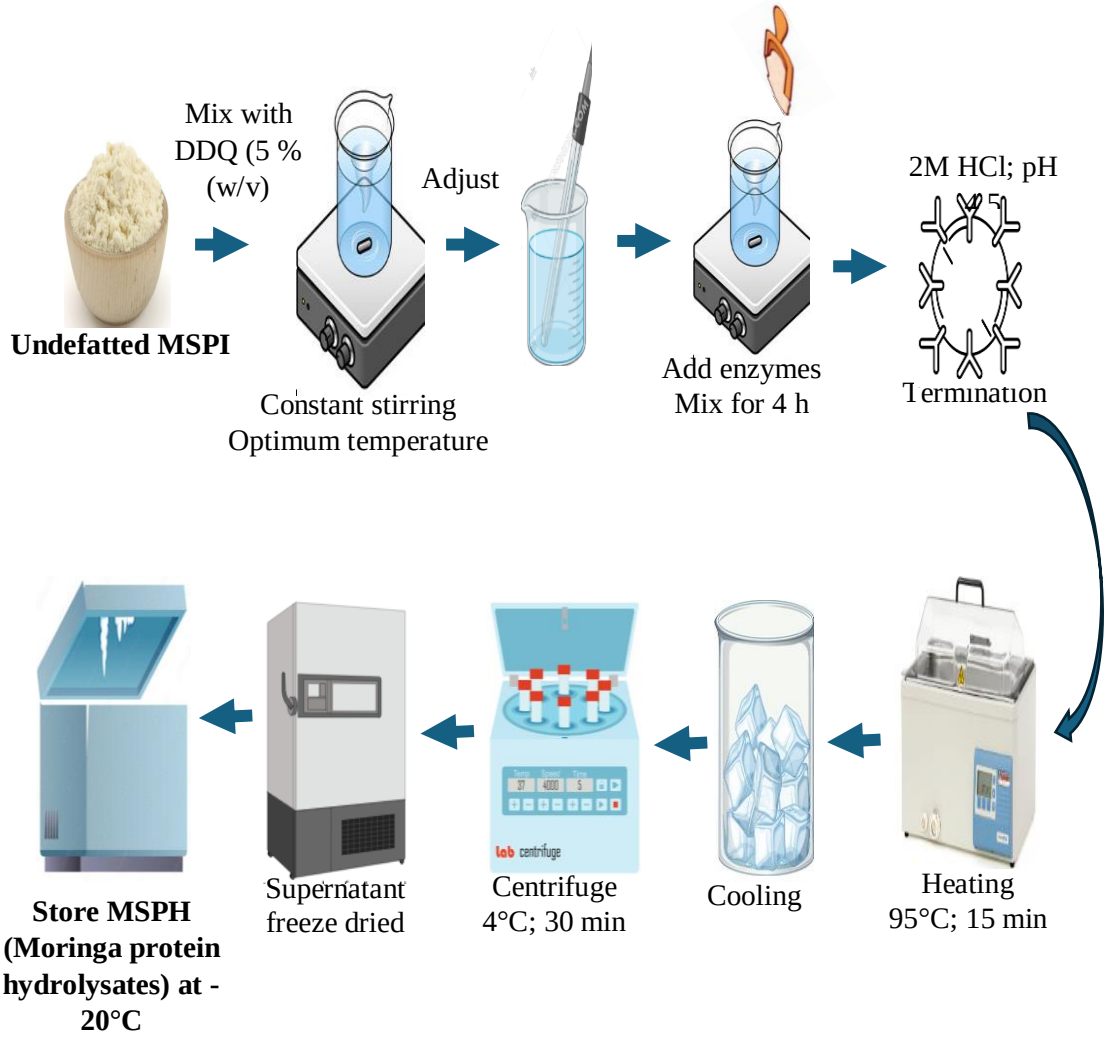

Figure 4. 1: Enzymatic hydrolysis of *Moringa stenopetala* protein isolate. Enzymes used and optimal conditions (Alcalase: pH 8.0; 50°C, Flavourzyme: pH 6.5; 50°C, Pepsin (pH 2.0) + pancreatin (pH 7.5); 37°C).

4.2.3 Protein content

The protein content of MSPH was determined using the Modified Lowry method as described by Markwell et al. (1978). To prepare a 10 mg/mL protein solution, 50 mg of the protein sample was dissolved in 5 ml of 0.1 M NaOH in a centrifuge tube and mixed with a vortex. The solution was allowed to hydrate for 1 hour, followed by centrifugation at 3600 rpm for 30 minutes. Serial dilutions (1 mg/mL to 10 mg/mL) were prepared from 1 ml of bovine serum albumin (BSA) or MSPH solutions. Reagent C was prepared at room temperature by combining 2% Na_2CO_3, 0.4% NaOH, 0.16% C_4O_6HKNa, 1% SDS, and 4% $CuSO_4$. Three milliliters of Reagent C were added to each test tube, mixed thoroughly with a vortex, and incubated for 1 hour in the dark. Subsequently, Reagent D was prepared by mixing equal parts of Folin-Ciocalteu reagent and DDW. After the first incubation, 0.3 ml of Reagent D was added to each tube, vortexed again, and incubated in the dark for an additional 45 minutes. Absorbance was measured at 660 nm using a UV spectrophotometer to quantify the protein concentration and total protein content. The total protein content was calculated using the following formulas:

$$Corresponding\ Conc\ of\ sample = \frac{Sample\ absorbance}{Standard\ absorbance} \times Standard\ concentration$$

$$\text{Protein content (\%)} = \frac{\text{Sample concentration}}{\text{Standard concentration}} \times 100$$

4.2.4 Amino acid composition

The amino acid composition of MSPH was evaluated using the HPLC Pico-Tag system, according to the method detailed by Bidlingmeyer et al. (1984). Sample hydrolysis was performed with 6 M HCl for 24 hours. Methionine and cysteine levels were quantified after oxidation with performic acid, following the protocol described by Gehrke et al. (1985). The tryptophan content was determined after alkaline hydrolysis, as per the method of Landry and Delhaye (1992).

4.2.5 Molecular weight distribution

The molecular weight distribution was analyzed using a GE AKTA system equipped with a Superdex Peptide 12 10/300 GL column (10 × 300 mm, GE, USA) and a UV detector set to 214 nm, as described by He et al. (2013). A 5 mg/mL protein sample was prepared in 50 mM phosphate buffer, pH 7.0 and containing 0.15 M NaCl. At room temperature, 100 µl of the sample was injected onto the column, and phosphate buffer was pumped through at a flow rate of 0.5 mL/min. The column was calibrated using standard reference compounds, including cytochrome (12,700 Da), aprotinin (6,512 Da), vitamin B12 (1,355 Da), and glycine (75 Da).

4.2.6 Surface hydrophobicity

The surface hydrophobicity of MSPH was assessed using the method described by Haskard & Li-Chan (1998), with 1-anilino-8-naphthalenesulfonate (ANS) as the fluorescent probe. A 10 mM phosphate buffer (pH 7.0) was prepared, and 10 mg of MSPH were dissolved in this buffer. The solution was stirred at room temperature for 1 h, then centrifuged at 7000 x g for 20 min. The supernatant was collected and diluted with 10 mM phosphate buffer (pH 7.0) to achieve protein concentrations ranging from 30 to 250 µg/mL. A 40 µl aliquot of 8 mM ANS was added to each 4.0 ml protein solution. The excitation wavelength was set at 390 nm and the emission wavelength at 470 nm using a spectrofluorometer (JASCO FP-6300). Fluorescence intensity (FI) was plotted against protein concentration (mg/mL), and protein surface hydrophobicity (So) determined as the slope of the linear regression line.

4.2.7 Angiotensin converting enzyme (ACE) inhibition

The ACE-inhibitory activities of the protein hydrolysates were evaluated following the method outlined by Alashi et al. (2013). The samples were dissolved in 50 mM Tris-HCl buffer,

pH 7.5 and containing 0.3 mM NaCl. To each sample, 10 µL of ACE solution (with an assay activity of 25 mU) was added to 50 µL of each peptide (1 – 5 mg/ml), along with 170 µL of 0.5 mM FAPPG, and the mixture incubated at 37 °C. The blank contained only the buffer, with no inhibitors. Captopril, an ACE-inhibitory drug, was used as a positive control, following a similar procedure. Absorbance was measured at 345 nm at 1-min intervals over a 30-min period to monitor the reaction rate. The percentage of ACE inhibition was calculated using the slopes of the absorbance curves for both the sample (SS) and the blank (SB), using the following equation:

$$\text{ACE inhibition (\%)} = \frac{(\text{SB} - \text{SS})}{(\text{SB})} \times 100$$

4.2.8 Renin inhibition

The renin-inhibitory activity of the protein hydrolysate samples was evaluated using the method described by Sonklin et al. (2020). In this procedure, 10 µL of distilled water was mixed with 20 µL of substrate and 150 µL of assay buffer, which was added to the background wells. The same mixture was prepared for the control wells (uninhibited) with the same volumes. For the sample wells (inhibited), 10 µL of the protein hydrolysate samples replaced the 10 µL of distilled water. Then, 10 µL of renin solution, dissolved in assay buffer, was added to both the control and sample wells to initiate the enzymatic reaction. The microplate was shaken for 10 seconds to ensure proper mixing, and the samples were incubated for 10 minutes at 37 °C in the dark. Fluorescence intensity (FI) was measured at an excitation wavelength of 340 nm and an emission wavelength of 490 nm to assess enzyme activity. Renin inhibition was calculated by subtracting the FI of the background wells from the FI of both the control (FI control) and sample (FI sample) wells. Renin inhibition was quantified using the following formula:

$$\text{Renin Inhibition (\%)} = \frac{\text{FI control} - \text{FI sample}}{\text{FI control}} \times 100$$

4.2.9 Arginase inhibition

Arginase inhibition was measured following the procedure described by Gao et al. (2021). The activity of arginases was assessed by colorimetric quantification of the produced urea. The assays were carried out in 96-well plates using an assay buffer consisting of 100 mM sodium phosphate (pH 7.4) and 130 mM sodium chloride. Each reaction had a final volume of 60 µL, with the final concentrations of arginase I (hargI) and arginase II (hargII) set at 50 nM per well. Samples without hargI or hargII served as negative controls. The peptide inhibitor was added at concentrations ranging from 1 to 10 mg/mL. Each sample was pre-incubated for 30 min in a shaking incubator at 37 °C (100 rpm), and the reactions were initiated by adding a concentrated substrate buffer containing 100 mM L-arginine, 500 mM glycine, and 1.25 mM $MnCl_2$ in the assay buffer. The reactions were conducted in the shaking incubator at 37 °C and stopped after 1 h by adding a stop mix. The stop mix consisted of two parts of freshly prepared solution A (antipyrine/H_2SO_4 reagent: 5 g/L of antipyrine in 50% (v/v) sulfuric acid) and one part of solution B (oxime reagent: 0.8 g of 2,3-butanedione monoxime in 100 mL of 5% (v/v) acetic acid). After stopping the reaction, the mixtures were allowed to develop color in the dark at room temperature for at least 18 h, followed by heating at 45 °C in a water bath for 4 h. The resulting brown color was quantified by measuring the optical density (OD) at 466 nm, which reflected urea production and served as an indicator of arginase activity.

4.2.10 α-amylase inhibition

The inhibition of α-amylase was assessed with slight modifications to the method outlined by Chiou et al. (2017). The samples were dissolved in 0.2 mM sodium phosphate buffer at pH 6.9

containing 6 mM NaCl. A 50 µL aliquot of the sample (concentrations ranging from 10 – 50 mg/mL) was mixed with 50 µL of α-amylase solution in a test tube, and the mixture incubated at 25 °C for 10 min. A 1% (w/v) starch solution was prepared by dissolving starch in the buffer, followed by heating and cooling. After the incubation, 50 µL of the starch solution was added to the sample mixture, and the reaction allowed to proceed for an additional 10 min at 25 °C. To terminate the enzyme reaction, 100 µL of 96 mM dinitrosalicylic acid (DNSA) prepared in 2 M sodium potassium tartrate tetrahydrate was added, and the mixture was heated in a water bath at 100 °C for 15 min. After cooling the reaction mixture to room temperature, an additional 1.5 mL of double-distilled water (DDW) was added. Subsequently, 200 µL of the final mixture was transferred to a 96-well microplate, and absorbance was measured at 540 nm at 25 °C using a SynergyTM H4 microplate reader (BiotekTM, Winooski, VT, USA). The absorbance of the blank (phosphate buffer) was subtracted from each well to calculate the enzyme activity. Acarbose, a known α-amylase inhibitor, was used as a standard and assayed under the same conditions as the samples. The α-amylase inhibitory activity was calculated using the following formula:

$$\alpha - \text{amylase Inhibition } (\%) = \frac{\text{C arb} - \text{S arb}}{\text{C arb}} \times 100$$

Where C arb is control absorbance and S arb is sample absorbance.

4.2.11 α-glucosidase inhibition

α-glucosidase inhibition analysis was conducted following the method adapted from Ranilla et al. (2010) with minor modifications, whereby 50 µL of peptides (concentrations ranging from 10 – 50 mg/mL) in 0.1 M sodium phosphate buffer at pH 6.9 were mixed with 50 µL of α-glucosidase solution in a 96-well microplate. The mixture was incubated at 37 °C for 10 min. After incubation, 100 µL of a 5 mM p-nitrophenyl α-D-glucopyranoside (PNP-glucoside) solution in phosphate buffer was added to each well. Absorbance was measured at 405 nm for 30 min at 60 s

intervals using a Synergy™ H4 microplate reader, with the temperature maintained at 37 °C. The absorbance from the blank (measured without the enzyme) was subtracted from the readings of each well. Acarbose, a known α-glucosidase inhibitor, was used as a standard and tested simultaneously with the samples. The α-glucosidase inhibitory activity was calculated using the following formula:

$$\alpha - \text{glucosidase Inhibition } (\%) = \frac{\text{C arb} - \text{S arb}}{\text{C arb}} \times 100$$

Where C arb is control absorbance and S arb is sample absorbance.

4.2.12 Pancreatic lipase inhibition

Pancreatic lipase activity was measured using a modified method based on Tang et al. (2016). Porcine pancreatic lipase (type II) and 4-methylumbelliferyl oleate (4-MUO) were used as the enzyme and fluorogenic substrate, respectively. In this procedure, 225 µL of 0.5 mM 4-MUO substrate and 25 µL of either peptide (10 – 50 mg/mL), orlistat standard (used as a positive control), or a blank were incubated at 37 °C for 15 min. After the incubation, 25 µL of enzyme solution (3.125 U/mL) was added to each well containing 13 mM Tris-HCl buffer, 150 mM NaCl, and 1.3 mM $CaCl_2$ (pH 8.0). The reaction continued at 37 °C for 1 h, and fluorescence intensity was measured as previously outlined. The lipase inhibitory activity was calculated as a percentage, with the absorbance of the blank subtracted from the readings of each well. The inhibitory activity of pancreatic lipase was then determined using the following formula:

$$\text{Pancreatic lipase Inhibition } (\%) = \frac{\text{C arb} - \text{S arb}}{\text{C arb}} \times 100$$

Where C arb is control absorbance and S arb is sample absorbance.

4.2.13 Trypsin inhibition

The inhibition of trypsin activity was assessed using a modified method based on the procedure described by Souza et al. (2016). In the presence of peptides, residual enzyme activity was measured using the substrate N-Benzoyl-L-arginine p-nitroanilide (BApNA) at pH 7.5. Trypsin (50 µL, at an assay concentration of 60 µg/mL), dissolved in Tris buffer at pH 7.5 with 0.02 M calcium chloride ($CaCl_2$), was used for the assay. Peptides (50 µL, concentrations ranging from 1–5 mg/mL) or buffer were added to the mixture, which was pre-incubated at 37 °C for 5 min. To activate the enzyme, 125 µL of a 1 mM BApNA solution prepared in 1% (v/v) dimethyl sulfoxide (DMSO) in Tris buffer was added. The reaction was carried out at 37 °C and terminated after 10 min by adding 25 µL of 30% (v/v) aqueous acetic acid. The extent of substrate hydrolysis was measured by absorbance at 410 nm. The absorbance of the blank was subtracted from the readings for each well. The inhibitory activity of trypsin was calculated using the following formula:

$$\text{Trypsin Inhibition (\%)} = \frac{\text{C arb} - \text{S arb}}{\text{C arb}} \times 100$$

Where C arb is control absorbance and S arb is sample absorbance.

4.2.13 Chymotrypsin inhibition

The inhibitory activity of chymotrypsin was assessed using a modified method based on El-Latif (2014), measured by the release of p-nitroaniline from the substrate benzoyl-L-tyrosine p-nitroanilide (BTpNA). Chymotrypsin (40 µg/mL) was dissolved in a 1 mM HCl solution containing 5 mM $CaCl_2$. A 50 mM Tris buffer containing 20 mM $CaCl_2$ (pH 8.2) was prepared. A total of 200 µL of peptides (1–5 mg/mL) was mixed with 500 µL of a 1 mM BTpNA solution prepared with 1% (v/v) dimethyl sulfoxide (DMSO) in Tris buffer, achieving a concentration of

0.2 mg/mL. This mixture was incubated at 37 °C for 15 min. Subsequently, 200 µL of chymotrypsin solution was added to the mixture, and the incubation continued at 37 °C for an additional 15 min. The reaction was stopped by adding 100 µL of 30% (v/v) acetic acid. Afterward, 200 µL of the final mixture was transferred to a 96-well microplate, and absorbance measured at 410 nm. Orlistat was used as a standard and tested following the same procedure as the samples. The absorbance of the blank was subtracted from the readings in each well. The percentage inhibitory activity of chymotrypsin was calculated using the following formula:

$$\text{Chymotrypsin Inhibition (\%)} = \frac{\text{C arb} - \text{S arb}}{\text{C arb}} \times 100$$

Where C arb is control absorbance and S arb is sample absorbance.

4.2.14 Acetylcholinesterase (AChE) inhibition

The activity of acetylcholinesterase (AChE) was evaluated using Ellman's colorimetric method with acetylthiocholine iodide (ATCI) as the substrate, based on a modified procedure from Khan and Ghani (2012). A 0.1 M phosphate buffer at pH 7.4 was prepared for the assay. In a microplate, 25 µL of each peptide solution or galantamine (assay concentrations of 2, 6, and 10 mg/mL) was mixed with 25 µL of the phosphate buffer, 25 µL of 15 mM ATCI in DDW, and 25 µL of AChE (0.5 U/mL). The mixture was incubated at 37 °C for 10 min. A blank control was set up using the same reagents, excluding the AChE enzyme. After incubation, 125 µL of 3 mM dithiobis (2-nitrobenzoic acid) (DTNB) dissolved in 50% ethanol was added to each well. Absorbance was recorded at 450 nm every minute for 10 min using a Synergy H4 multi-mode microplate reader, also maintained at 37 °C. The blank absorbance values were subtracted from the sample readings, and the percentage of AChE inhibition was calculated using the formula:

$$\text{AChE Inhibition (\%)} = \frac{\text{C arb} - \text{S arb}}{\text{C arb}} \times 100$$

Where C arb is control absorbance and S arb is sample absorbance.

4.2.15 DPPH radical scavenging activity (DRSA)

The DRSA was assessed following the method outlined by Olarewaju et al. (2018). The experiment was conducted in a 96-well clear flat-bottom microplate. Peptide solutions were prepared in 0.1 M sodium phosphate buffer (pH 7.0) containing 1% (w/v) Triton X-100 to achieve assay concentrations of 0.5, 2.5, and 5 mg/mL. For the blank, DPPH was dissolved in methanol to reach a final concentration of 100 µM. In the microplate, 100 µL of the peptide solution was combined with 100 µL of the DPPH solution and incubated in the dark at room temperature for 30 min. Absorbance readings for both the blank and sample solutions were taken at 517 nm using a Synergy H4 multi-mode microplate reader, with glutathione (GSH) serving as the standard. The DRSA was expressed as a percentage, calculated using the following formula:

$$\text{DRSA (\%)} = \frac{\text{B arb} - \text{S arb}}{\text{B arb}} \times 100$$

Where B arb is blank absorbance and S arb is sample absorbance.

4.2.16 Superoxide radical scavenging activity (SRSA)

The SRSA was measured following the procedure described by Famuwagun et al. (2020). A solution of each peptide (80 µL, 1–10 mg/mL) was combined with 80 µL of 50 mM Tris–HCl buffer (pH 8.3) containing 1 mM EDTA in a clear-bottom 96-well plate. To initiate the reaction, 40 µL of 1.5 mM pyrogallol prepared in 10 mM HCl was added to each well. The reaction rate (R) for each sample (R sample) was determined by measuring the absorbance increase at 420 nm over 4 min at room temperature. A blank control (R blank) was prepared using the Tris–HCl buffer but without the protein fraction. The percentage of SRSA was calculated using the following formula:

$$\text{SRSA (\%)} = \frac{\text{R blank} - \text{R sample}}{\text{R blank}} \times 100$$

Where R blank is blank reaction rate and R sample is sample reaction rate.

4.2.17 Hydroxyl radical scavenging activity (HRSA)

The HRSA was evaluated using the method described by Kumari and Ra (2017). MSPH and 3 mM 1,10-phenanthroline were dissolved separately in a 0.1 M sodium phosphate buffer (pH 7.4), while 3 mM FeSO4 and 0.01% (v/v) hydrogen peroxide were prepared in deionized distilled water (DDW). In a clear, flat-bottom 96-well plate, 50 µL of peptide solution (assay concentrations of 0.25, 1.25, and 2.5 mg/mL) or buffer (as a blank) was combined with 50 µL of 1,10-phenanthroline and 50 µL of FeSO4. The Fenton reaction was initiated by adding 50 µL of hydrogen peroxide, and the plate was then covered and incubated at 37°C for 1 h with shaking. Absorbance readings were taken at 536 nm at 10-min intervals during the incubation period using a spectrophotometer. The HRSA was determined based on the reaction rate (R) and calculated using the following formula:

$$\text{HRSA (\%)} = \frac{\text{R blank} - \text{R sample}}{\text{R blank}} \times 100$$

Where R blank is blank reaction rate and R sample is sample reaction rate.

4.2.18 Ferric reducing antioxidant power (FRAP)

The FRAP of the peptides was measured using a modified version of the method described by Olarewaju et al. (2018). To prepare the FRAP reagent, a mixture of 300 mM acetate buffer (pH 3.6), 10 mM 4,6-tripyridyl-s-triazine (TPTZ) dissolved in 40 mM hydrochloric acid (HCl), and 20 mM ferric chloride was combined in a ratio of 5:1:1. In a 96-well microplate, 40 µL of each peptide solution (assay concentrations of 0.6, 0.8, and 1.6 mg/mL) was added to the FRAP reagent.

Absorbance was recorded at 593 nm using a Synergy H4 multimode microplate reader. Results were expressed in millimoles of ferric ion (Fe^{3+}) reduced to ferrous (Fe^{2+}) per gram of peptides, calculated using a calibration curve prepared with ferrous sulfate heptahydrate (25–150 mM). Glutathione (GSH) served as the positive control and was analyzed using the same procedure.

4.2.19 Metal chelation activity (MCA)

Metal chelation activity was determined following a modified method from Asen et al. (2021). In this assay, 500 µL of peptide solution or GSH (concentrations ranging from 1 to 10 mg/mL) was mixed with 25 µL of 2 mM $FeCl_2$ and 925 µL of deionized distilled water (DDW) in a reaction tube. A 50 µL aliquot of 5 mM ferrozine solution was then added, and the solution mixed thoroughly. After a 10 min incubation at room temperature, 200 µL of the reaction mixture was transferred to a 96-well plate. A blank reaction was prepared by replacing the peptide solution with 500 µL of DDW. Absorbance was measured at 562 nm using a spectrophotometer. The percentage of metal chelation activity was calculated using the following formula:

$$\text{Metal chelating activity (\%)} = \frac{\text{C blk} - \text{S arb}}{\text{C blk}} \times 100$$

Where C blk is blank absorbance and S arb is sample absorbance.

4.2.20 Inhibition of linoleic acid peroxidation

The method described by Li et al. (2008) was used to measure linoleic acid oxidation. Peptides (10 µL final volume) were dissolved in 2.5 mL of 0.1 M phosphate buffer (pH 7.0) and mixed with 2.5 mL of 50 mM linoleic acid in a glass test tube. The mixture was stored in the dark at 60°C for 7 days. At 24-h intervals, 100 µL samples were collected and combined with 0.1 mL of 30% (w/v) ammonium thiocyanate, 0.1 mL of 0.02 M $FeCl_2$ in 1 M HCl, and 4.7 mL of 75%

aqueous ethanol. After 3 min, the absorbance of each mixture was recorded at 500 nm using a Synergy H4 multi-mode microplate reader. Glutathione (GSH) was used as a standard.

4.2.21 Statistical analysis

All assays were performed in triplicate, and mean values with standard deviations were calculated. Statistical analysis was conducted using one-way analysis of variance (ANOVA). Duncan's multiple range test was employed to identify significant differences between means at a 5% significance level. The analyses were performed using IBM SPSS Statistics software (version 24, Armonk, NY, USA) and GraphPad Prism (version 9.0, GraphPad Software, San Diego, CA, USA).

4.3 Results and discussion

4.3.1 Hydrolyzed Protein yield and protein content of MSPH

The protein yield and content of MSPH as shown in Table 4.1, exceeded 50%, indicating that the proteases used were effective in breaking down the proteins into peptides. This suggests that *Moringa stenopetala* seed proteins are highly susceptible to enzymatic hydrolysis, which is beneficial for their economic potential in food applications and nutraceuticals. The protein yield ranged from 61.92% to 86.23%, with Alcalase hydrolysates (ALH) producing the lowest yield and pepsin + pancreatin hydrolysates (PPH) generating the highest yield. However, protein content followed a different trend, where ALH resulted in the highest protein content (72.16%) and PPH produced the lowest (68.32%). The protein yield and content observed for ALH in this study are similar to those reported for canola protein hydrolysates, which achieved yields of 66.75% and protein content of 75.80%, as noted by Alashi et al. (2014). This finding is further supported by López-Pedrouso et al. (2020), who demonstrated that Alcalase hydrolysis of porcine liver

produced primarily small peptides, whereas hydrolysis with Flavourzyme resulted in a greater proportion of free amino acids.

Alcalase, being an endoprotease, specifically hydrolyzes peptide bonds adjacent to hydrophobic residues like leucine, isoleucine, and valine (Kaur, 2024). This selective cleavage limits the extent of protein breakdown, resulting in fewer smaller peptides, which remain largely in the soluble fraction after centrifugation. Consequently, the protein yield might be lower, but the higher proportion of intact proteins contributes to a higher protein concentration in the final product. In contrast, the combined action of pepsin + pancreatin is more thorough. Pepsin targets aromatic amino acids (Abimbola et al., 2017), while pancreatin, with its exopeptidase activity, breaks down peptides at terminal ends, producing free amino acids (Alashi et al., 2014). This extensive breakdown leads to a higher protein yield, but free amino acids, which are generally not included in protein content measurements, contribute to a lower overall protein content. Flavourzyme, with both endopeptidase and exopeptidase activities, breaks down proteins from within the peptide chain and at terminal ends, efficiently reducing proteins into smaller peptides and free amino acids (Tacias-Pascacio et al., 2020). Its dual action results in moderate protein yields, but the formation of shorter peptides and free amino acids reduces the overall intact protein content, as these smaller fragments are harder to quantify during protein content analysis. Additionally, free amino acids are not typically included in protein content measurements, further lowering the final content.

Table 4 1: Protein content, protein yield, and surface hydrophobicity of protein hydrolysate obtained from *Moringa stenopetala* seed meal hydrolyzed with alcalase (ALH), flavourzyme (FLH), and pepsin + pancreatin (PPH)

Samples	Protein content (%)	Protein yield (%)	Surface hydrophobicity
ALH	72.16 ± 0.42^{a}	61.92 ± 0.83^{c}	7.61 ± 0.27a
FLH	70.06 ± 0.71^{b}	71.70 ± 1.27^{b}	0.45 ± 0.12c
PPH	68.32 ± 0.11^{c}	86.23 ± 0.53^{a}	0.61 ± 0.06b

ALH – Alcalase hydrolysate, PPH – Pepsin + pancreatin hydrolysate, FLH – Flavourzyme hydrolysate. Each value represents the mean ± standard deviation from three independent measurements. Different superscript letters (a, b, c,) within a column denote significant differences ($p < 0.05$).

4.3.2 Surface hydrophobicity of MSPH

When proteins undergo breakdown through proteolysis, their previously concealed hydrophobic regions become exposed. Hydrophobicity plays a critical role in determining the effectiveness of antioxidant peptides, as these regions help stabilize the 3D structure and mediate interactions with other proteins and cell membranes (Liang et al., 2023). The biological functions of peptides are driven by hydrophobic interactions, and understanding these properties offer valuable information about the peptides' mechanisms of action. (Mundi & Aluko, 2014). Studies have shown that peptides containing amino acids with highly hydrophobic or aromatic side chains tend to exhibit strong inhibition of ACE activity, indicating that hydrophobic traits significantly influence their biological activity (Mundi & Aluko, 2014).

As shown in Table 4.1, ALH resulted in the highest surface hydrophobicity, likely due to its extensive hydrolysis, which exposes more hydrophobic regions. Additionally, alcalase cleaves near hydrophobic amino acids, further increasing surface hydrophobicity. As Rezvankhah et al. (2021) observed, alcalase specifically targets peptide bonds close to hydrophobic amino acids, leading to greater exposure during hydrolysis. In contrast, FLH had the lowest surface hydrophobicity of 0.45. This may be due to flavourzyme’s specificity, which limits extensive hydrolysis, as indicated by the higher molecular weight of FLH. Consequently, fewer hydrophobic regions are exposed compared to ALH. This finding is consistent with Li et al. (2024), who reported that peptides with lower molecular weights tend to have higher surface hydrophobicity than those with higher molecular weights. The increase in hydrophobicity is largely attributed to the exposure of hydrophobic amino acids as large proteins are broken down into smaller peptides. These smaller peptides are more effective in interacting with hydrophobic environments, such as cell membranes, which enhances their biological functionality (Rezvankhah et al 2021).

4.3.3 Amino acid composition of MSPH

Table 4.2 presents the amino acid profiles of MSPH. These profiles are essential for understanding the potential nutritional and functional applications, as well as the antioxidant properties of the MS protein hydrolysates (Aderinola et al., 2018). The results showed an increase in amino acid values following protein hydrolysis compared to the isolates, with enzymatic treatment influencing the distribution of specific amino acids. For instance, GLX (glutamic acid + glutamine) were the most abundant amino acid across all treatments, ranging from 14.88% for PPH to 16.66% for FLH, with FLH having the highest concentration. The high content of GLX in FLH suggests that its hydrolysates may have strong umami flavor potential, enhancing its application in flavor enhancement in food. The study by Aderinola et al. (2018) also reported GLX as the most abundant amino acid in Alcalase, Pepsin, and Trypsin hydrolysates from *Moringa oleifera* seeds, although the GLX values in this study are superior to those reported.

The second most abundant amino acid across all hydrolysates was Arg, an amino acid with significant health benefits, including cardiovascular protection (Yuan et al., 2022). ARG showed high contents, particularly in FLH (10.48%), followed by ALH (9.90%) and PPH (9.46%). This might contribute to the antihypertensive properties of the protein hydrolysates. ALH produced hydrolysates with a slightly higher EAA content (34.04%), making it a preferred enzyme for generating nutritionally balanced protein products and more suitable for nutraceutical applications. On the other hand, FLH exhibited a higher content of NEAA (47.46%).

The varying profiles of aromatic (AAA) and sulfur-containing (SCAA) amino acids suggest that these hydrolysates could offer diverse health benefits, including antioxidant activities and flavor enhancement. Since plants are generally low in SCAA (Cys and Met), their content was relatively low across all treatments, with the lowest value being 2.85 % for PPH and the highest

value being 3.04% for FLH. However, the presence of these amino acids is crucial as they play roles in antioxidant mechanisms and protein structure. The concentration of Cys is similar to the values reported for red lionfish protein hydrolysates using pepsin and pancreatin (Chuc-Koyoc et al., 2024). Proline, an imino acid crucial for contributing to peptide ACE-inhibitory activity, was reported by Idowu et al. (2021) for sesame seed hydrolysate with pepsin-pancreatin as 3.85%, which is lower than the values obtained in this study, ranging from 4.19% to 4.41%, with FLH having the highest. This result is also compared with the values reported for Bambara protein Alcalase hydrolysates and canola protein Alcalase hydrolysates, which are 4.3% and 5.6%, respectively (Alashi et al., 2014; Arise et al., 2017).

Leucine (LEU) and Isoleucine (ILE), both branched-chain amino acids (BCAAs) essential for synthesizing muscle protein, were higher in ALH. HAA (Hydrophobic Amino Acids) showed substantial representation across all treatments, with values ranging between 27.68% and 29.15% for PPH and ALH, respectively. This observation corresponds with Arise et al. (2017) report, who explained that Bambara protein hydrolysates obtained from Alcalase had the highest HAA compared to those from pepsin and trypsin. This claim was also backed by Aderinola et al. (2018), who reported higher HAA values for Alcalase *Moringa oleifera* seed protein hydrolysates. These amino acids are crucial for interactions within hydrophobic environments, influencing protein structure and stability. ALH produced the highest concentration of AAA. The values reported by Idowu et al. (2021) for tryptophan, phenylalanine, and tyrosine in sesame seed protein hydrolysates obtained with pepsin-pancreatin are superior to the findings in this study.

Table 4 2: Amino acid composition of protein hydrolysate obtained from *Moringa stenopetala* seed meal hydrolyzed with alcalase (ALH), flavourzyme (FLH), and pepsin + pancreatin (PPH)

Amino acids	ISO	ALH (%)	PPH (%)	FLH (%)
Amm	1.16 ± 0.04	1.01 ± 0.10	0.98 ± 0.05	1.09 ± 0.02
His	1.53 ± 0.12	2.24 ± 0.06	2.10 ± 0.02	2.36 ± 0.14
Ser	1.85 ± 0.00	2.14 ± 0.03	2.10 ± 0.02	2.10 ± 0.02
Arg	9.14 ± 0.21	9.90 ± 0.18	9.46 ± 0.02	10.48 ± 0.01
Gly	2.62 ± 0.06	3.63 ± 0.09	3.45 ± 0.02	3.40 ± 0.01
Asx	4.13 ± 0.02	4.57 ± 0.11	4.49 ± 0.12	4.26 ± 0.05
Glx	14.43 ± 0.19	16.32 ± 0.12	14.88 ± 0.31	16.66 ± 0.08
Thr	1.74 ± 0.05	2.03 ± 0.02	1.92 ± 0.00	1.90 ± 0.01
Ala	2.71 ± 0.03	3.33 ± 0.03	3.18 ± 0.04	3.13 ± 0.02
Pro	3.74 ± 0.07	4.36 ± 0.04	4.19 ± 0.04	4.41 ± 0.04
Cys	0.81 ± 0.03	1.19 ± 0.01	1.22 ± 0.02	1.37 ± 0.05
Lys	1.18 ± 0.02	1.36 ± 0.00	1.44 ± 0.04	1.26 ± 0.01
Tyr	1.41 ± 0.04	1.76 ± 0.01	1.73 ± 0.01	1.64 ± 0.05
Met	1.25 ± 0.04	1.79 ± 0.04	1.62 ± 0.01	1.67 ± 0.09
Val	3.27 ± 0.01	4.01 ± 0.04	3.72 ± 0.02	3.73 ± 0.02
Ile	2.48 ± 0.00	2.78 ± 0.06	2.69 ± 0.04	2.69 ± 0.00
Leu	4.66 ± 0.08	5.35 ± 0.06	4.96 ± 0.02	5.13 ± 0.00
Phe	3.15 ± 0.06	3.69 ± 0.08	3.42 ± 0.02	3.40 ± 0.01
Trp	0.93 ± 0.00	0.88 ± 0.00	0.96 ± 0.01	0.76 ± 0.02
HAA	24.42	29.15	27.68	27.93

PCAA	2.71	3.60	3.54	3.62
NCAA	18.56	20.89	19.37	20.92
AAA	5.59	6.33	6.10	5.80
SCAA	2.06	2.98	2.85	3.04
BCAA	7.15	8.14	7.65	7.82
EAA	29.32	34.04	32.28	33.38
NEAA	40.84	47.20	44.68	47.46

ISO – Protein isolates obtained from undefatted *Moringa stenopetala* meal (Alkaline solubilization followed by isoelectric precipitation), ALH – Alcalase hydrolysate, PPH – Pepsin + pancreatin hydrolysate, FLH – Flavourzyme hydrolysate. HAA - hydrophobic amino acids (alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine, and cysteine), PCAA - positively charged amino acids (histidine, lysine), NCAA- negatively charged amino acids (Asx - asparagine + aspartic acid) and (Glx - glutamine + glutamic acid), AAA - aromatic amino acids (phenylalanine, tryptophan, and tyrosine), SCAA - sulphur containing amino acids (cysteine and methionine), BCAA - branched chain amino acids (leucine and isoleucine), EAA - essential amino acids (threonine, valine, methionine, isoleucine, leucine, phenylalanine, histidine, lysine, arginine, and tryptophan), NEAA – non-essential amino acids (alanine, arginine, Asp (asparagine + aspartic acid), cysteine, Glu (glutamic acid + glutamine), glycine, proline, serine, and tyrosine). Each value represents the mean ± standard deviation from two independent measurements.

4.3.4 Molecular weight distribution of MSPH

As shown in Figure 4.2, we observed distinct differences in the molecular weight distribution across the hydrolysates. These variations in elution profiles reflect the unique specificity and proteolytic activity of each enzyme, as evidenced by the major and secondary peaks observed across the chromatogram. During the initial elution phase (0-10 mL), all three hydrolysates exhibit consistently low absorbance, indicating the absence of significant amounts of larger peptides, which would typically elute earlier in size exclusion chromatography. This suggests a predominance of smaller peptides in the samples.

The highest peak for FLH occurred at an elution volume of 16 mL, representing a 6.89 kDa peptide. This is the largest peptide size observed among all hydrolysates, but it's important to note that FLH's (0.72 - 6.89 kDa) overall peaks are lower compared to PPH (0.41 - 2.37 kDa) and ALH (0.38 - 2.19 kDa). The presence of a high molecular weight peptide here reflects Flavourzyme's combined endopeptidase and exopeptidase activities, which allows it to target internal peptide bonds while also trimming terminal residues (Gurumallesh et al., 2019). However, it shows less efficiency in breaking down proteins into smaller fragments compared to the other enzymes. FLH presented two additional peaks at 18 mL (2.21 kDa) and 20 mL (0.72 kDa). These represent smaller peptide fragments, but their lower absorbance suggests limited production of smaller peptides. The relatively modest production of these low molecular weight peptides highlights Flavourzyme's specialization in producing larger peptides that are broken down to smaller ones more slowly. PPH shows a strong peak at 18 mL, corresponding to a 2.37 kDa peptide, overlapping with the ALH peak at the same elution volume. Pepsin, with its specificity for aromatic amino acids, initiates the digestion process by generating mid-sized peptides, while pancreatin further breaks down these fragments. This overlapping peak reflects the efficient mid-sized peptide production of both

enzymes, though PPH generates slightly larger peptides than ALH at this stage. PPH produces low molecular weight (LMW) peptides, with secondary peaks between 20-21 mL, representing 0.68 kDa and 0.41 kDa peptides. These lower molecular weight peptides reflect pancreatin's ability to further break down mid-sized peptides into smaller fragments.

The major peak for ALH appeared at 18 mL with an absorbance corresponding to a 2.19 kDa peptide, slightly lower than PPH but still significant. Alcalase, known for its serine endoprotease activity, cleaves peptide bonds near hydrophobic amino acids, generating mid-sized peptides. This strong peak reflects Alcalase's efficiency in hydrolyzing proteins into moderately sized fragments. ALH also produces smaller molecular weight peptides, as indicated by peaks between 20 and 21 mL. The smallest peptides detected among all the hydrolysates have molecular weights of 0.38 kDa, showing that Alcalase efficiently breaks down proteins into LMW peptides. This is crucial, as LMW peptides are typically associated with bioactive properties such as antioxidants or antihypertensive activities. The lower molecular weight observed for ALH indicates its proficiency in producing peptides with potential bioactivity, which could be advantageous in nutraceutical applications. Arise et al. (2017) reported that the LMW peptides possess better ACE inhibitory activities when in comparison with HMW peptides. It was also reported that Bambara groundnut Alcalase hydrolysates were found to have a larger range of LMW peptides compared to pepsin and trypsin. Similarly, Anh et al. (2020) reported that Alcalase hydrolysates of soybean proteins yielded the lowest molecular weight peptides compared to Flavourzyme hydrolysates.

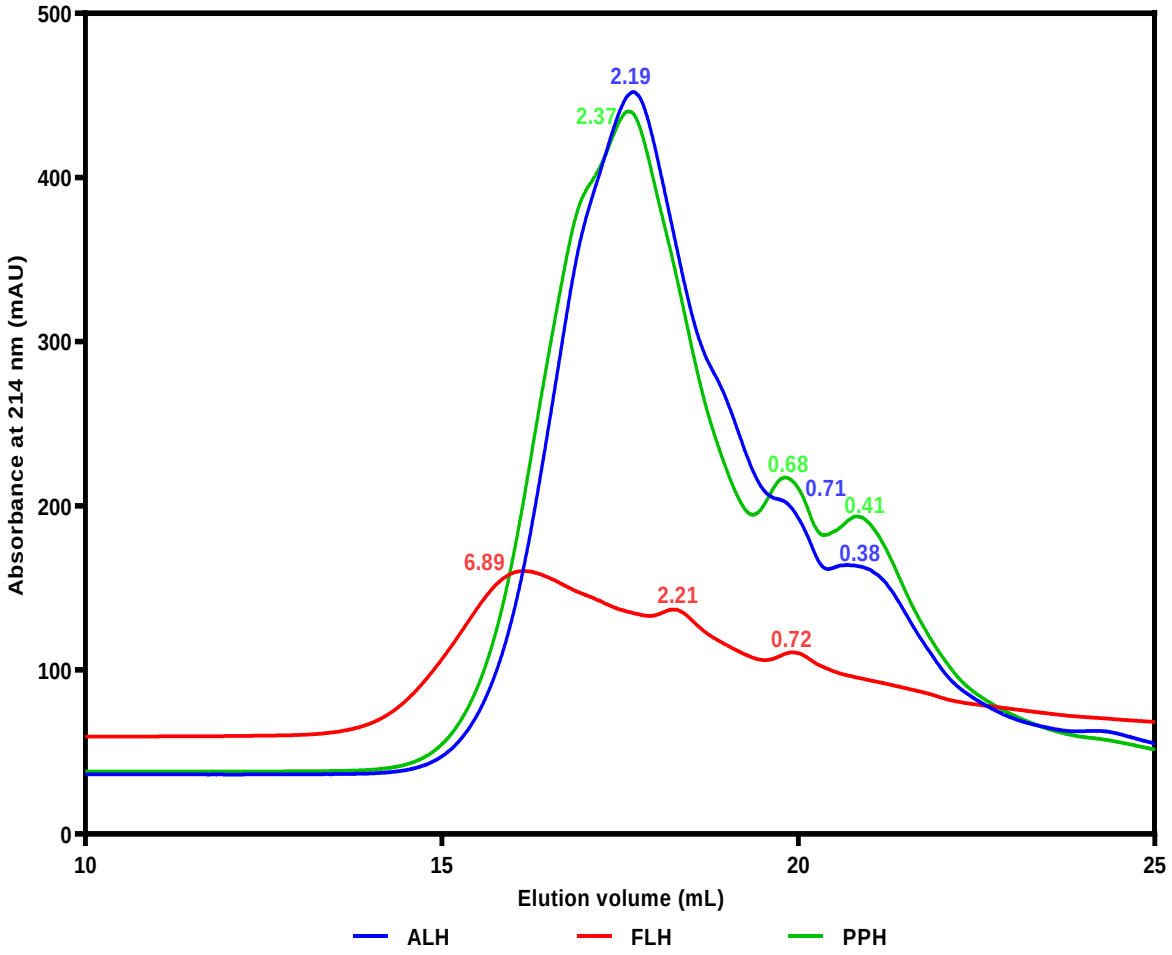

Figure 4. 2: Molecular weight distribution of *Moringa stenopetala* protein hydrolysates. ALH – Alcalase hydrolysate, PPH – Pepsin + pancreatin hydrolysate, FLH – Flavourzyme hydrolysate.

4.3.5 Angiotensin converting enzyme (ACE) inhibitory activity

Captopril, the control, demonstrated consistently high ACE inhibition across all tested concentrations, indicating its effectiveness even at lower doses (Figure 4.4A). Its activity remained relatively stable regardless of concentration, underscoring its potency. In contrast, all hydrolysates showed a concentration-driven increase in ACE-inhibitory activity, with higher concentrations leading to enhanced effectiveness. Notably, FLH exhibited no ACE inhibitory activity at the lowest concentration of 0.2 mg/mL. The ACE inhibitory activity of PPH in this study is lower compared to the findings reported by Olagunju et al. (2022), which showed pepsin-pancreatin hydrolysates derived from pigeon pea protein had an ACE inhibition activity of 61.82% at 0.2 mg/mL. This is also lower than the activity observed for *Moringa oleifera* alcalase hydrolysates reported by Aderinola et al. (2018).

Conversely, at 0.2 mg/mL, sesame protein pepsin-pancreatic hydrolysates demonstrated ACE-inhibitory activity similar to that of PPH at the same concentration, as noted in Idowu et al. (2021). The ACE-inhibitory activity observed in this study is also comparable to the findings of Xia et al. (2022), which reported approximately 50% for pea protein hydrolysates at 1 mg/mL. Among all the proteases tested, ALH exhibited the highest activity (15.07% - 66.56%) across all concentrations, followed by PPH. Similarly, Xie et al. (2019) reported that alcalase-derived mung bean hydrolysate had the best ACE-inhibitory activity among various proteases, as also supported by Arise et al. (2017), which found that Bambara groundnut alcalase protein hydrolysate showed the highest inhibitory activity against ACE among the tested proteases.

The increased activity observed in ALH may be attributed to the higher amounts of leucine, isoleucine, and proline, which, according to Tacias-Pascacio et al. (2020), contribute to the increased ACE inhibitory activity of peptides. Additionally, ALH and PPH contained low

molecular weight (LMW) peptides, with ALH having the lowest MW peptides, potentially enhancing its effectiveness in inhibiting ACE activity. This might be because lower molecular weight peptides, being smaller and more flexible, can navigate more easily into the active site, enhancing their potential to inhibit ACE effectively. This variation in size enables smaller peptides to interact more effectively with the enzyme, enhancing their biological activity. Furthermore, ALH's low molecular weight, along with its high concentration of hydrophobic amino acids and surface hydrophobicity, likely contributed to its potent ACE-inhibitory effect. Mirzaee et al. (2023) reported enhanced ACE inhibitory properties in soy proteins with high hydrophobicity. Coreas (2022) further explained that hydrophobic characteristics facilitate the passage of substances through the bi-lipid cell membrane, promoting cellular entry and improving targeting to specific organs.

4.3.6 Renin-inhibitory activity

Renin is an enzyme that is secreted by the kidneys and helps in regulating blood pressure (Girgih et al., 2011). Its main function is to break down angiotensinogen, a glycoprotein primarily produced by the liver, into angiotensin I, an inactive molecule (Alashi et al., 2014). Once angiotensin I is formed, ACE converts it into angiotensin II, primarily in the lungs (Turner & Nalivaeva, 2022). Angiotensin II acts as a powerful vasoconstrictor, causing the narrowing of blood vessels, which results in an elevation in blood pressure (Onuh et al., 2016). Additionally, angiotensin II triggers the release of aldosterone, a hormone that prompts the kidneys to retain more sodium and water, further elevating blood pressure by increasing blood volume (Funder, 2017).

Elevated renin activity is a contributing factor in the pathogenesis of hypertension, underscoring the importance of compounds that can inhibit this enzyme and serve as

antihypertensive agents (Idowu et al., 2021). Figure 4.4B presents the renin inhibition activity of MSPH at three different concentrations (0.2 to 1.2 mg/mL). The specificity of each enzyme plays a critical role in determining the types and sizes of peptides produced during hydrolysis, which subsequently impacts their renin-inhibitory potential. The inhibitory activities of all hydrolysates demonstrated a concentration-dependent increase. ALH also exhibited the highest renin inhibition across all concentrations, likely attributable to the presence of LMW peptides, and more hydrophobic residues. In contrast, PPH showed slightly lower inhibition at the lowest concentration compared to ALH; however, at higher concentrations, PPH's inhibition approached that of ALH. This indicates that the peptides generated from PPH may still possess significant renin-inhibitory activity when present at increased concentrations.

FLH exhibited the lowest overall renin inhibition, ranging from 10.74% at 0.2 mg/mL to 34.66% at 1.0 mg/mL, which was statistically significant ($p < 0.05$) when compared to ALH. This finding contrasts with previous research (Malomo et al., 2015) that reported renin inhibition levels of approximately 65% and 70% for hemp protein hydrolysates produced with Alcalase and pepsin-pancreatin, respectively, at a concentration of 0.2 mg/mL. The observed differences may be attributed to variations in sample composition and amino acid profiles. Moreover, the aforementioned study indicated that hemp protein hydrolysates exhibited higher renin inhibition compared to ACE-inhibition. This contradicts the results of the present study, where the renin-inhibitory activity of MSPH was weaker than their ACE-inhibition. Our findings align with previous reports (Alashi et al., 2014) for canola protein hydrolysates and (Girgih et al., 2011) for hemp seed protein hydrolysates, which also noted higher ACE inhibition relative to renin inhibition.

4.3.7 Arginase inhibitory activity of MSPH

Arginase is essential in the urea cycle, where it converts L-arginine into L-ornithine and urea. In the context of hypertension, inhibiting arginase is crucial because of its impact on nitric oxide (NO) production (Caldwell et al., 2015). Both arginase and nitric oxide synthase (NOS), the enzyme responsible for NO synthesis, use L-arginine as a common substrate (Clemente et al., 2020). NO acts as a potent vasodilator, relaxing blood vessels and reducing blood pressure (Pudlo et al., 2017). Elevated arginase activity competes with NOS for L-arginine, reducing NO production (Niu et al., 2022). This decline can lead to vasoconstriction, contributing to hypertension (Aluko, 2012). By inhibiting arginase, more L-arginine is made available for NO synthesis, improving vascular health and blood pressure regulation. Arginase inhibitors thus hold potential as therapeutic agents for hypertension by increasing NO levels and enhancing vascular functions.

Figure 4.4C illustrates the arginase inhibitory activity of MSPH, showing a concentration-dependent effect similar to findings on ACE and renin inhibition. All hydrolysates exhibited increased inhibition at higher sample concentrations. At lower concentrations, PPH showed weaker inhibition, likely due to peptides that are less efficient at inhibiting arginase at these levels. Conversely, FLH displayed the highest inhibition, especially at 2 mg/mL, significantly outperforming ALH and PPH. This superior activity may be linked to FLH's significantly ($p < 0.05$) higher arginine content compared to ALH and PPH. Since arginase and NOS compete for L-arginine, FLH not only inhibits arginase but could also potentially increase L-arginine availability for NO production, amplifying its vasodilatory effects and improving vascular health. Supporting this, Onuh et al. (2016) demonstrated that a diet supplemented with 1% casein soy protein

hydrolysate improved NO availability in hypertensive rats by enhancing L-arginine levels, ensuring efficient NO production by NOS.

The enhanced arginase inhibition activity of FLH can also be attributed to its high SCAA content, particularly Cys and Met. Srivastava et al. (2010) associated elevated SCAA levels with stronger arginase inhibition. Sulfur-containing amino acids impact arginase activity by modulating the redox state and influencing NO pathways (Castro & Kim, 2020). As arginase competes with NOS for L-arginine, cysteine-rich peptides can indirectly boost NO levels by suppressing arginase activity (Tang & Le, 2016). Furthermore, the thiol group (-SH) in cysteine residues may interact with arginase's active sites, altering enzyme conformation, inhibiting its activity, or functioning as a competitive inhibitor, depending on peptide structure and composition (Tejero et al., 2019).

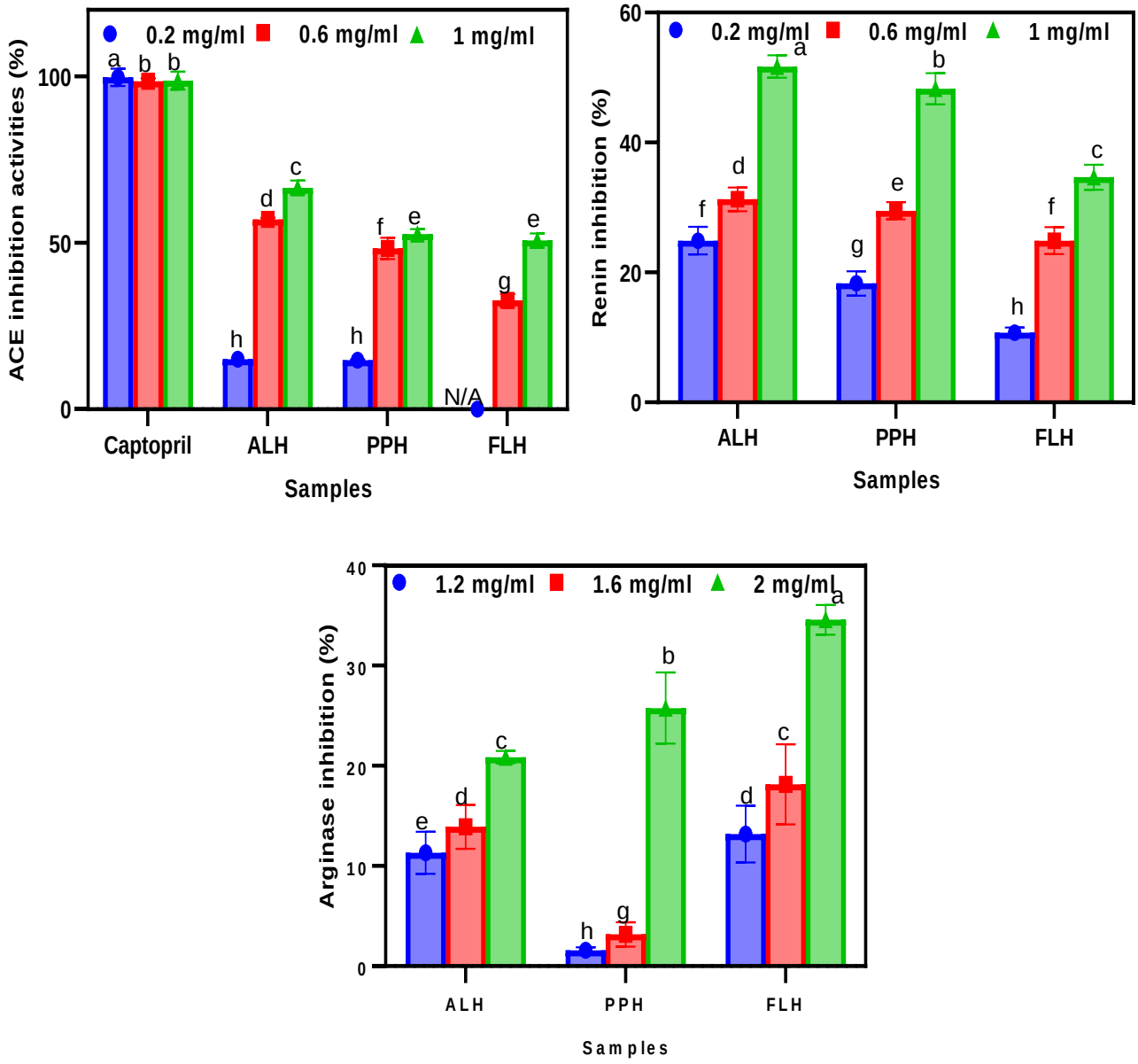

Figure 4. 3: ACE (A), renin (B), and arginase (C) inhibitory activity of MSPH. ALH – Alcalase hydrolysate, PPH – Pepsin + pancreatin hydrolysate, FLH – Flavourzyme hydrolysate. Each value represents the mean ± standard deviation from three independent measurements. Different superscript letters denote significant differences ($p < 0.05$).

4.3.8 α-amylase inhibitory activity

One of the primary strategies for managing diabetes involves slowing glucose absorption by inhibiting α-amylase, one of the enzymes responsible for carbohydrate breakdown (Bassogog et al., 2024). This reduction in monosaccharide absorption helps control postprandial hyperglycemia (Toma et al., 2014). For ALH and FLH, α-amylase inhibitory activity decreased with increasing concentration, while for PPH, it increased with concentration. Among all hydrolysates, FLH consistently showed the lowest inhibitory activity across all concentrations. The α-amylase inhibitory activities of the hydrolysates, as shown in Figure 4.5A, were notably significant, particularly for ALH and PPH, which exhibited inhibition rates ranging from 86% to 100%. At most concentrations, the inhibitory activity of these hydrolysates did not differ significantly ($p < 0.05$) from the standard drug acarbose (97%), with no notable variation in acarbose's activity across all concentrations. Both ALH and PPH exhibited significantly ($p < 0.05$) higher α-amylase inhibitory activities compared to FLH, likely due to their low molecular weights. This observation aligns with the report by (Toledo et al. 2016) and (Mirzapour-Kouhdasht et al., 2022), which indicated that LMW peptides exhibit higher α-amylase inhibition than HMW peptides. A previous study reported lower inhibitory activity against α-amylase for *Moringa oleifera* seed hydrolysates (Ferahtia, 2021). Another study showed that the α-amylase inhibitory activity of wild lettuce protein isolate, as reported by Ijarotimi et al. (2021), was lower than the values observed in this study for all hydrolysates, except at the highest concentration for FLH.

4.3.9 α-glucosidase inhibitory activity

α-Glucosidase is an enzyme found in the brush border of the small intestine, where it plays a vital role in starch digestion (Toma et al., 2016). It breaks down carbohydrates into monosaccharides, such as glucose, which are then readily absorbed by the body (Olagunju et al.,

2021). Inhibiting glucose absorption in the intestine is a useful strategy for managing postprandial hyperglycemia in diabetic patients (Toma et al., 2016). When comparing the α-amylase inhibitory activities of the hydrolysates, α-glucosidase inhibition ranged from 5% to 18%, significantly lower than that of acarbose (Figure 4.5B). No significant difference was observed in the activity of the control across all concentrations. The activity of ALH decreased as concentration increased, eventually showing no inhibition at the highest concentration. Similarly, PPH activity also declined at higher concentrations, although it exhibited the highest overall activity among the hydrolysates. In contrast, FLH activity increased with concentration, reaching its maximum at the highest concentration among all the hydrolysates. These findings are lower than those reported by Olagunju et al. (2021) for pigeon pea protein hydrolysates and by Ijarotimi et al. (2021) for African wild lettuce protein isolate, but higher than the results reported by Aderinola et al. (2022) for *Moringa oleifera* seed hydrolysates.

4.3.10 Pancreatic lipase inhibitory activity

The results presented in Figure 4.5C illustrate the effectiveness of MSPH in inhibiting the enzyme pancreatic lipase, also known as triacylglycerol acyl hydrolase. This enzyme is essential for fat digestion, as it breaks down dietary fats (triglycerides) into monoacylglycerols, diacyglycerols, and free fatty acids, which are then absorbed through the walls of the digestive tract (Esfandi et al., 2023). Inhibiting pancreatic lipase slows the digestion of fats, leading to reduced fat absorption, which can help in managing obesity (Lammi et al., 2015: Ajayi et al., 2021). Therefore, evaluating the potential of MSPH as pancreatic lipase inhibitors is important since effective inhibitors may serve as nutraceuticals or therapeutic agents for obesity management. In comparison, Orlistat, a known lipase inhibitor, exhibited the highest lipase inhibitory activity, approaching 100% across all concentrations tested. Among the MSPHs, the

hydrolysates displayed inhibitory potential, though their activity generally decreased as the concentration increased.

FLH exhibited the highest inhibitory activity at the lowest concentration tested, while PPH showed moderate activity, and ALH demonstrated the lowest inhibitory activity across all concentrations. At 5 mg/mL, the results were comparable to those reported by Toma et al. (2014) for the pancreatic lipase inhibitory activity of *Moringa stenopetala* leaf extract at the same concentration (Toma et al., 2014). Notably, at the lowest concentration (1 mg/mL), both PPH and FLH showed over 50% inhibition, indicating that these hydrolysates are particularly effective at lower concentrations. In comparison with the results from a previous research work on the pancreatic lipase inhibitory activity of protein hydrolysates derived from legumes such as green pea, chickpea, black bean, lentil, and fava bean, the activity of alcalase-treated green pea hydrolysates was comparable, while black bean hydrolysates demonstrated superior inhibition (Moreno, et al., 2020). Moreover, the PPH activity observed in this study surpassed that of pepsin-pancreatin-treated chickpea, lentil, and fava bean hydrolysates. These variation in the results may be due to variations in the bioactive compounds found in each hydrolysate.

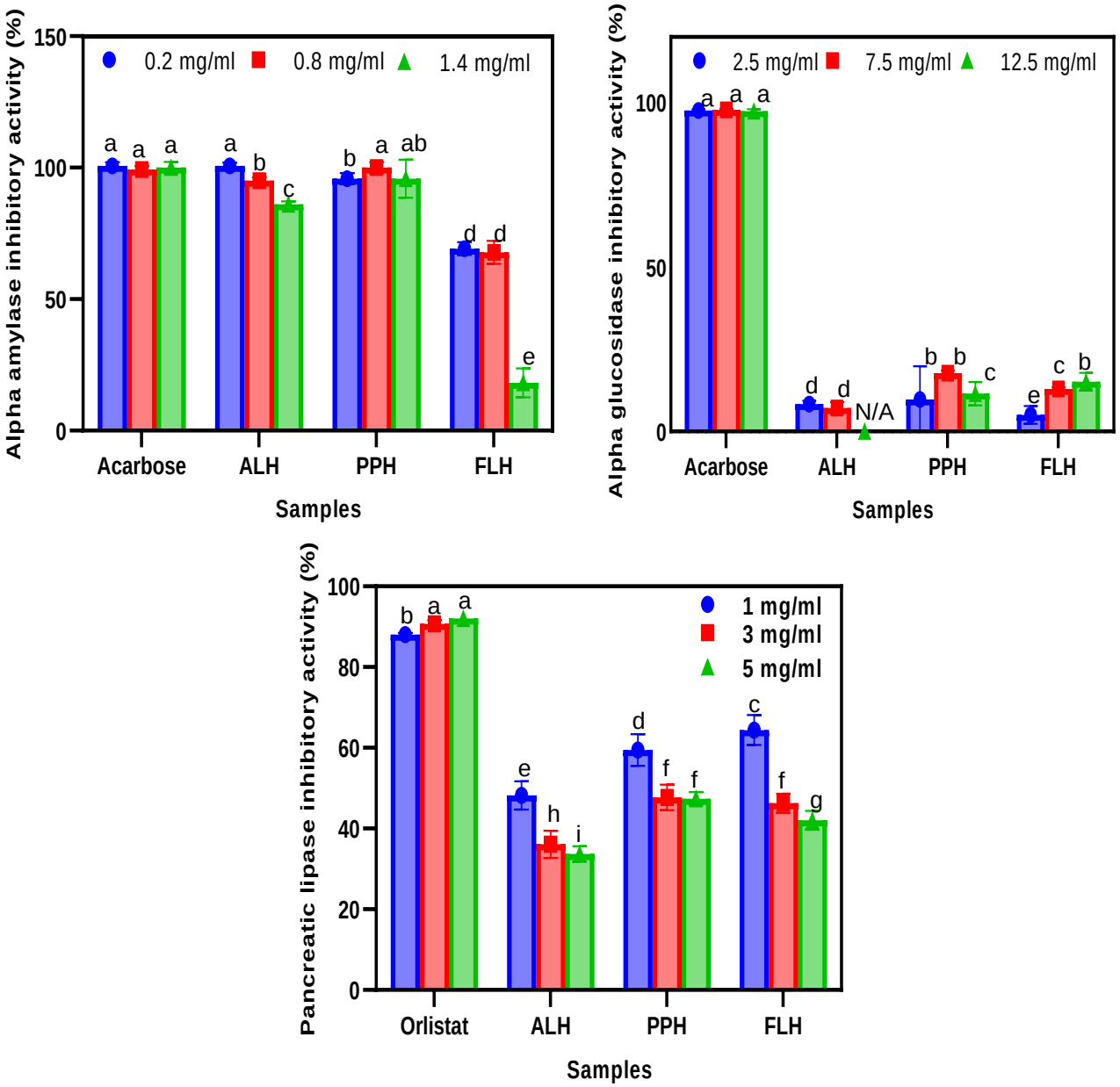

Figure 4. 4: α-amylase (A), α-glucosidase (B), and pancreatic lipase (C) inhibitory activity of *Moringa stenopetala* seed protein hydrolysates. ALH – Alcalase hydrolysate, PPH – Pepsin + pancreatin hydrolysate, FLH – Flavourzyme hydrolysate. Each value represents the mean ± standard deviation from three independent measurements. Different superscript letters denote significant differences ($p < 0.05$).

4.3.11 Trypsin inhibitory activity of MSPH

The results presented in Figure 4.5A demonstrate the effectiveness of MSPH in inhibiting the digestive enzyme trypsin, a crucial protease involved in the digestion of dietary proteins. Trypsin specifically targets peptide bonds formed by the carboxyl groups of arginine and lysine residues (Ahmed et al., 2022). In the small intestine, it cleaves these bonds, resulting in smaller peptides and amino acids that are absorbed into the bloodstream (Ahmed et al., 2022). By inhibiting trypsin activity, MSPH can disrupt this normal digestion process, limiting protein breakdown and potentially reducing protein absorption. This inhibition has important implications, such as the ability to modulate protein digestion for therapeutic purposes such as anti-viral agents or to create functional food applications where controlling protein breakdown is beneficial (Li-Chan et al., 2015). For instance, trypsin inhibitors could be used in conditions where reduced protein digestion is desired, such as certain metabolic disorders, or in nutraceutical formulations aimed at slowing protein absorption for extended satiety (McClements, 2018).

Orlistat, a well-known trypsin inhibitor, displayed the highest trypsin inhibitory activity at all concentrations tested. However, its activity decreased as the concentration was lowered. In contrast, the trypsin inhibitory activity of MSPH increased with rising concentrations. Among the hydrolysates, ALH exhibited the strongest trypsin inhibition, while FLH showed the lowest activity across all concentrations. These results diverge from previous findings, such as those of Lammi et al. (2015), who reported lower trypsin inhibitory activity for alcalase-treated pea protein hydrolysates. However, our findings surpass those of Choi et al. (2019), who observed lower trypsin inhibitory activity in various legumes, including soybean, mung bean, adzuki bean, and chickpea. This indicates that MSPH, particularly the ALH, could offer superior inhibition of

trypsin compared to other plant protein sources, highlighting its potential in nutraceutical and functional food development.

4.3.12 Chymotrypsin inhibitory activity

Chymotrypsin is a crucial enzyme in the small intestine that aids in protein digestion. It specifically targets peptide bonds linked to aromatic amino acids, such as phenylalanine, tyrosine, and tryptophan (Ahmed et al., 2022). In collaboration with trypsin, chymotrypsin facilitates the thorough breakdown of dietary proteins into smaller peptides and amino acids, enabling their absorption by the body (Do, 2017). By inhibiting chymotrypsin, the digestion of proteins slows down, which can reduce the availability of amino acids. This mechanism could be strategically used to regulate protein digestion in scenarios where protein restriction or slower digestion is required, such as in certain digestive disorders, or to promote gut health (Jahan-Mihan et al., 2011). Additionally, modulating chymotrypsin activity could enhance the delivery of bioactive peptides in functional food products (Jahan-Mihan et al., 2011; McClements, 2018). In the current study, all samples, including orlistat, demonstrated an increase in chymotrypsin inhibitory activity as concentrations rose. The control consistently showed the highest level of inhibition across all concentrations (Figure 4.5B).

Among the hydrolysates, PPH exhibited the strongest chymotrypsin inhibition at higher concentrations, while ALH and FLH showed no significant differences in activity across all concentrations ($p < 0.05$). The chymotrypsin inhibitory activity observed in this study aligns with the findings of Awosika and Aluko (2019), which reported increased inhibition of chymotrypsin with higher concentrations in pea protein hydrolysates. However, their study reported a maximum activity of 48.13%, which was significantly higher than the 24.93% observed in our study. It is worth noting that their maximum activity was measured at a concentration of 6 mg/mL, whereas

our study achieved comparable inhibition at a much lower concentration of 1 mg/mL. In comparison to chymotrypsin inhibition, MSPH showed stronger trypsin inhibitory activity, consistent with findings from a study that reported about 50% inhibition of trypsin but no inhibition of chymotrypsin in xoconostle seed protein hydrolysates (Aguirrezabala-Cámpano et al., 2013). This suggests that the specificity of the enzymes used in hydrolyzing proteins might explain the variation in inhibitory activities, with ALH showing higher inhibition against trypsin and PPH demonstrating stronger inhibition against chymotrypsin. This variation may be due to the different protease specificities in targeting peptide bonds within proteins during digestion.

4.3.13 Acetylcholinesterase inhibitory

Acetylcholinesterase (AChE) plays a crucial role in regulating nerve impulses by breaking down the neurotransmitter acetylcholine (ACh) into choline and acetic acid at cholinergic synapses (Bowirrat Abdalla, 2016). It is an extremely efficient enzyme, capable of hydrolyzing around 5000 molecules of ACh per second (Spontón et al., 2016). The inhibition of AChE is an important area of research due to its implications for treating neurological disorders, especially Alzheimer's disease (AD) (Asen et al., 2023). AD is characterized by cognitive decline and memory loss due to the death of cholinergic neurons, leading to reduced ACh levels in the brain (Kumar and Balaji, 2022). According to the cholinergic hypothesis, maintaining or increasing ACh levels can slow cognitive decline in AD patients (Kumar and Balaji, 2022). AChE inhibitors work by reducing the breakdown of ACh, thereby increasing its concentration in the brain. These inhibitors mimic ACh's structure and bind to the enzyme's active site, reducing the enzyme's catalytic efficiency (Natarajan et al., 2013). This approach has shown therapeutic benefits in treating AD, as well as other neurological disorders like Parkinson's disease (Natarajan et al., 2013).

AChE inhibitors not only improve cognitive function but also help alleviate neuropsychiatric symptoms in AD patients (Shah et al., 2023). Figure 4.5C presents the AChE inhibitory activities of MSPH. The control, galantamine, showed the highest AChE inhibition across all concentrations, with its activity increasing proportionally with concentration. Similarly, the AChE inhibition of all hydrolysates increased as their concentrations rose. Among the hydrolysates, ALH and FLH displayed moderate and comparable inhibitory activities at the highest concentration of 10 mg/mL, achieving approximately 50% inhibition at this level. In contrast, PPH exhibited the lowest AChE activity. These findings are consistent with the AChE inhibition reported for Candida blankie trypsin hydrolysate by (Spontón et al., 2016), although our results surpass those for Saccharomyces cerevisiae pepsin hydrolysate. However, the AChE inhibitory activity observed in this study is lower than that reported for hemp protein hydrolysate (Malomo, 2015).

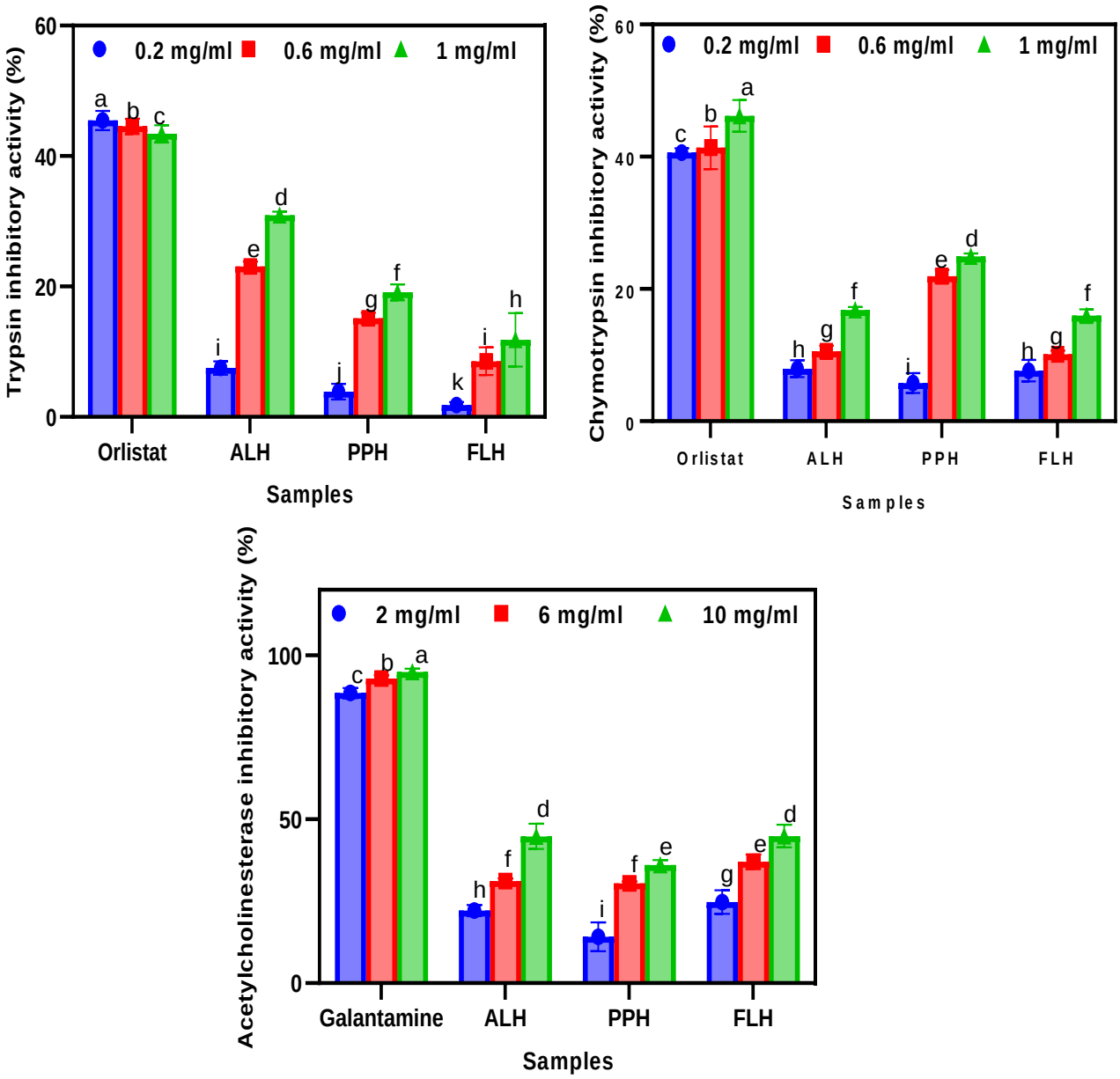

Figure 4. 5: Trypsin (A), chymotrypsin (B), and AChE (C) inhibitory activity of *Moringa stenopetala* seed protein hydrolysates. ALH – Alcalase hydrolysate, PPH – Pepsin + pancreatin hydrolysate, FLH – Flavourzyme hydrolysate. Each value represents the mean ± standard deviation from three independent measurements. Different superscript letters denote significant differences ($p < 0.05$).

4.3.14 DPPH radical scavenging activity (DRSA)

DPPH is a stable free radical capable of accepting an electron or hydrogen atom to form a stable, non-reactive molecule. The DRSA assay is a common method for assessing the antioxidant potential of various protein hydrolysates (Bassogog et al., 2024). Figure 4.6A illustrates the DRSA of MSPH and glutathione (GSH), which served as the positive control due to its strong antioxidant properties as a cellular bioactive peptide. Composed of cysteine, glutamic acid, and glycine, GSH neutralizes free radicals by donating protons (Sonklin et al., 2018). In this study, PPH and FLH displayed greater DRSA when compared to ALH. Interestingly, a study by Aderinola et al. (2019) similarly reported that *Moringa oleifera* alcalase hydrolysates had the lowest DPPH radical scavenging activity when compared to pepsin and trypsin hydrolysates. However, the current findings show higher DRSA for MS alcalase hydrolysates than those reported by Bassogog et al. (2024) for *Moringa oleifera* alcalase hydrolysates. No significant difference ($p < 0.05$) was observed between the DRSA of PPH and FLH at 2.5 mg/ml.

ALH showed decreasing DRSA with increasing peptide concentration, eventually exhibiting no activity at the highest concentration tested. In contrast, FLH demonstrated a concentration-dependent increase, with the highest activity recorded at 5 mg/ml among the hydrolysates. This surpasses the DRSA of flavourzyme hydrolysates derived from fish protein, as reported by Alahmad et al. (2023). Nonetheless, all hydrolysates had lower DRSA when compared to GSH. Zou et al. (2016) noted that low molecular weight peptides tend to have higher DRSA, while Girgih et al. (2015) highlighted a positive correlation between hydrophobic amino acids and DRSA. However, this trend was not observed in the present study. Despite its larger molecular weight and lower hydrophobic amino acid content compared to ALH, FLH exhibited the highest DRSA. Chen et al. (2018) proposed that factors such as higher concentrations of amino acids like

His, Val, and Glu could enhance DPPH activity. Notably, FLH contained elevated levels of His and Glu, which may have contributed to its superior antioxidant performance.

4.3.15 Superoxide radical scavenging activity (SRSA)

Superoxides are reactive molecules generated in the body during normal physiological processes (Nwachukwu & Aluko, 2019). As members of the reactive oxygen species (ROS) family, they pose a risk of cellular damage if not neutralized, primarily because they act as precursors to highly reactive species like hydroxyl radicals and hydrogen peroxide (Nwachukwu & Aluko, 2018). Figure 4.6B illustrates the SRSA of MSPH, showing a marked increase in activity with rising concentrations. This pattern is consistent with the findings of Sonklin et al. (2018), who observed a similar concentration-dependent increase in scavenging activity for mungbean protein hydrolysates. In this study, the hydrolysates displayed SRSA ranging from 18.50 % to 84.55 %, with PPH demonstrating the highest activity at all concentrations tested. These results contrast with the significantly lower activities (12% to 34.4%) reported by Aderinola et al. (2022) for alcalase- and trypsin-derived hydrolysates from Moringa oleifera seed proteins ($p < 0.05$). Aderinola et al. (2022) also noted that smaller peptides tend to exhibit stronger SRSA than larger peptides. Interestingly, in the present study, PPH, which contains smaller peptides (next to ALH), showed the highest SRSA. This suggests that peptide size may play a pivotal role in enhancing scavenging capacity, as smaller peptides in PPH appear to be more effective at neutralizing superoxide radicals when compared to larger peptides. However, this finding also suggests that factors beyond peptide size, such as amino acid composition, peptide length, and sequence specificity, may influence scavenging activity, warranting further exploration.

4.3.16 Hydroxyl radical scavenging activity (HRSA)

The reaction between Fe^{2+} and H_2O_2 produces hydroxyl radicals, highly reactive molecules that can damage vital cellular components (Sonklin et al., 2018). Oxidation of biological macromolecules often results in degradation, impairing cell functions, and contributing to chronic diseases such as heart disease or cancer through sustained oxidative damage (Chigurupati et al., 2017). As illustrated in Figure 4.6C, the HRSA of ALH at 0.25 mg/mL was significantly higher than that of GSH. At this concentration, ALH demonstrated the highest scavenging activity among all hydrolysates, likely due to its low molecular weight. Similar findings were reported by Nwachukwu & Aluko (2019) for low molecular weight soybean peptides, and He et al. (2013) for rapeseed peptides with stronger scavenging activity.

Interestingly, as the concentration of ALH increased, its scavenging activity declined, showing no activity at the highest concentrations (1.25 mg/mL and 2.5 mg/mL). This suggests that ALH is most effective at lower concentrations, while higher levels may induce antagonistic effects that diminish its hydroxyl radical scavenging capacity. Conversely, FLH displayed an opposing trend, with its scavenging activity peaking at higher concentrations. At 0.25 mg/mL, FLH appeared to be at a suboptimal concentration for effective scavenging, but its activity improved as the concentration increased. Despite this, the scavenging activity of FLH in this study was lower compared to fish protein flavourzyme hydrolysates reported by Alahmad et al. (2023). Beyond molecular weight, the amino acid composition of the peptide chain may have also played a role in potentiating the HRSA. For example, PPH showed its strongest activity at the highest concentration, while displaying the weakest activity at 1.25 mg/mL. Interestingly, FLH demonstrated a steady increase in activity with increasing concentrations, indicating that HRSA is influenced not only by low molecular weight but also by the specific arrangement of amino acids

in the peptide chain (Malomo et al., 2020). The results obtained in this study are superior to those reported for *Moringa oleifera* protein isolate at 1 mg/mL (Aderinola et al., 2019).

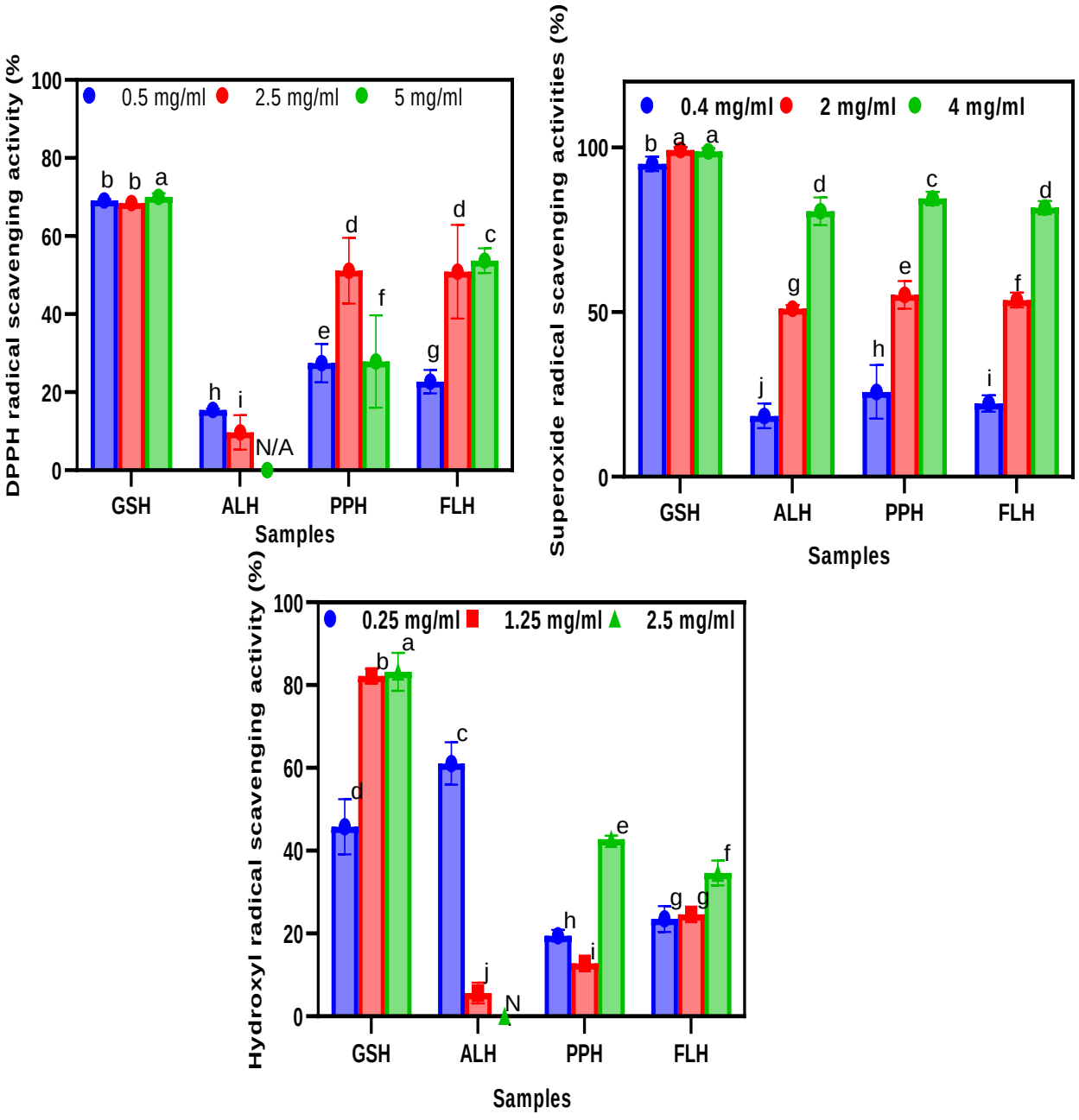

Figure 4. 6: DPPH (A), superoxide (B), and hydroxyl (C) radical scavenging activity of *Moringa stenopetala* seed protein hydrolysates. ALH – Alcalase hydrolysate, PPH – Pepsin + pancreatin

hydrolysate, FLH – Flavourzyme hydrolysate. Each value represents the mean ± standard deviation from three independent measurements. Different superscript letters denote significant differences ($p < 0.05$).

4.3.17 Ferric reducing antioxidant power (FRAP)

Figure 4.7A illustrates the FRAP of MSPH at three different concentrations. The FRAP assay evaluates a compound's ability to donate electrons, which facilitates the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) (Hunsakul et al., 2022). All hydrolysates, including GSH, demonstrated a concentration-dependent increase in FRAP. This indicates that higher concentrations of peptides enhance their capacity to reduce ferric ions. Specifically, GSH exhibited notable FRAP, with its activity increasing alongside concentration. At a concentration of 1.6 mg/mL, GSH reached its highest FRAP value, peaking at approximately 2.25 mmol. In comparison, the hydrolysates exhibited lower FRAP than GSH. Among them, PPH showed the highest antioxidant activity across all tested concentrations, although there was no significant difference in activity between PPH and the FLH at the lowest concentration. The results from this study align with previous findings, such as those reported by Olagunju et al. (2022), which noted comparable FRAP values for pigeon pea pepsin-pancreatin protein hydrolysate. Kimatu et al. (2017) also found similar antioxidant activities for hydrolysates derived from mushroom protein using alcalase, flavorzyme, and pancreatin at a concentration of 1 mg/mL.. However, Malomo et al. (2020) reported superior activity for pepsin-derived cashew nut and alcalase-derived fluted-pumpkin protein hydrolysates at a concentration of 2 mg/mL.

4.3.18 Metal chelation activity (MCA)

The results presented in Figure 4.7B underscores the potential of MSPH for use in applications where metal ion chelation is critical in preventing oxidative damage. Metal ions like

Fe^{2+} and Cu^{2+} are highly reactive, capable of converting superoxide radicals into hydroxyl radicals. These hydroxyl radicals are extremely reactive and can cause significant damage to vital cellular components such as proteins and DNA (Nwachukwu & Aluko, 2019). Bioactive peptides that can chelate these metal ions, forming stable complexes to prevent harmful reactions, hold promise in reducing oxidative damage, which is a contributing factor to long-term health conditions like heart disease and cancer (Nwachukwu & Aluko, 2019). The chelation activity of peptides is thought to be linked to their amino acid composition, allowing them to interact with metal ions such as Fe^{2+} (Kim et al., 2018). In the study, GSH showed no detectable MCA at the lowest tested concentration, while the hydrolysates exhibited significantly higher MCA at this same concentration. All hydrolysates demonstrated chelating ability at the lowest concentration, though this activity was absent at higher concentrations. Among the hydrolysates, PPH had the highest activity at the lowest concentration, while ALH and FLH showed similar levels of activity. This indicates that these peptides may have some potential for metal ion chelation at lower concentrations, which could contribute to their antioxidant effects. In contrast, GSH may not be effective in this regard at lower concentrations. A similar observation was made by Kim et al. (2018), who reported that GSH did not exhibit Fe^{2+} chelation activity at 0.3 mg/mL, while sea squirt protein hydrolysate demonstrated superior activity at this concentration compared to the findings in this study.

4.3.19 Linoleic acid peroxidation inhibitory activity

Lipid peroxidation is the oxidative degradation of lipids, which occurs when they are exposed to oxygen, resulting in the formation of harmful byproducts known as peroxides. These peroxides can cause significant damage to biological cells and food systems, leading to deterioration and compromised integrity (Nwachukwu & Aluko, 2019). Specifically, lipid

peroxidation disrupts the cell membrane, which plays a vital role in protecting the cell and maintaining its internal environment, potentially leading to cellular dysfunction (He et al., 2017). Peptides, with their antioxidant properties, can mitigate oxidative damage by inhibiting lipid peroxidation. Their ability to reduce lipid oxidation, particularly in a linoleic acid model system, serves as an indicator of their effectiveness as antioxidants (Sohaib et al., 2017). Figure 4.7C illustrates the inhibition of lipid peroxidation by *Moringa stenopetala* seed protein hydrolysates. The blank (control), which lacks any inhibition, exhibits a marked increase in absorbance at 500 nm over the course of seven days, reflecting substantial linoleic acid peroxidation. In contrast, GSH (glutathione) demonstrated strong antioxidant activity, maintaining a low absorbance with only a gradual rise, indicating its effectiveness in preventing peroxidation. ALH, PPH, and FLH followed similar trends with minimal increases in absorbance, showing that they effectively inhibited the peroxidation of linoleic acid. Among the hydrolysates, PPH stands out for having an inhibition profile most similar to that of GSH, reflecting high antioxidant potential. However, ALH and FLH exhibited the strongest inhibition overall, with trends that surpass both GSH and PPH, demonstrating their superior antioxidant capacity. The bioactive peptides present in these hydrolysates likely interact with peroxyl radicals generated during linoleic acid peroxidation. They may function by donating hydrogen atoms or electrons to neutralize these radicals, thereby interrupting the chain reaction that drives lipid peroxidation. This finding is consistent with a previous study (Arise et al., 2017), which reported that alcalase hydrolysates of Bambara groundnut were more effective over seven days compared to pepsin and trypsin hydrolysates, exhibiting a similar trend to the present study. On the other hand, research by Kimatu et al. (2017) on mushroom protein hydrolysates observed that their inhibition lasted up to day four, with a loss of activity on day five. This decrease in activity was attributed to the gradual loss of the

hydrolysates' ability to neutralize linoleic peroxyl radicals during extended incubation, as lipid peroxidation slowly progressed in the system.

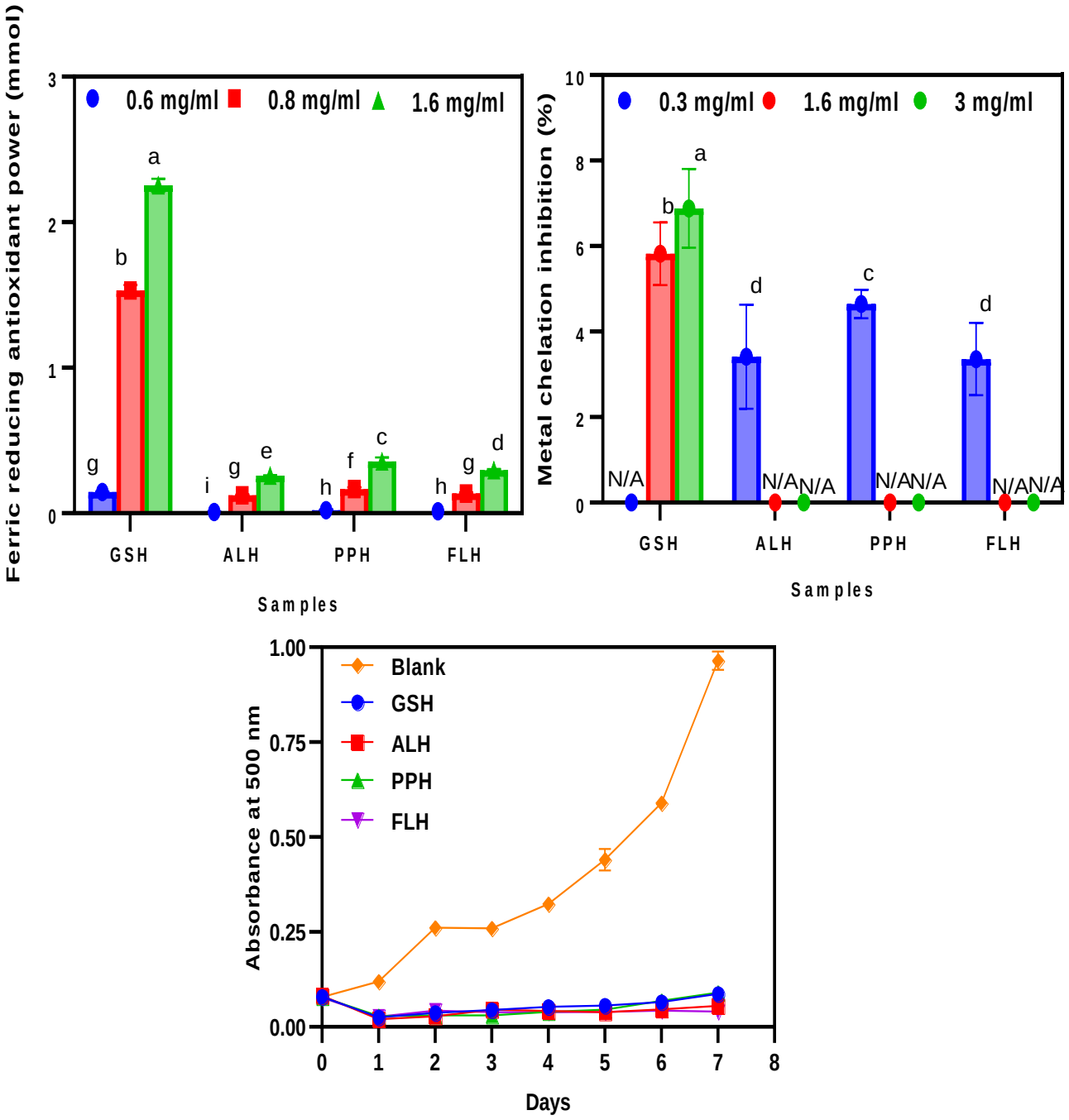

Figure 4. 7: Ferric reducing antioxidant power (A), metal chelation activity (B), and linoleic acid peroxidation (C) inhibitory activity of *Moringa stenopetala* seed protein hydrolysates. ALH – Alcalase hydrolysate, PPH – Pepsin + pancreatin hydrolysate, FLH – Flavourzyme hydrolysate.

Each value represents the mean ± standard deviation from three independent measurements. Different superscript letters denote significant differences ($p < 0.05$).

4.4 Conclusion

In summary, bioactive peptides derived from MS seeds showed great potential for developing functional foods with therapeutic benefits. Enzymatic hydrolysis of MS seed proteins generated peptides with notable health-promoting properties, including antihypertensive, antioxidant, and antidiabetic effects, making them ideal for use in nutraceutical formulations. This chapter emphasizes the strong antihypertensive activity of hydrolysates produced with Alcalase, which could be potentially effective in hypertension management. ALH demonstrated the highest protein content and surface hydrophobicity among the samples, alongside a balanced amino acid profile enriched with hydrophobic, aromatic, and essential amino acids. These results suggest that the roles of individual amino acids within peptide sequences may be more critical than their collective effects in determining the bioactive properties of MSPH. PPH achieved the highest protein yield, making it highly efficient for protein recovery. While ALH and PPH generally outperformed FLH in most evaluations, FLH displayed stronger inhibitory activity against arginase and pancreatic lipase, suggesting its potential for applications targeting these enzymes. As consumer interest in natural, health-enhancing food products continues to rise, MS seeds offer a promising avenue for innovative nutraceuticals aimed at improving human health and well-being. The findings presented here enhance the understanding of MS-derived peptides' therapeutic potential and pave the way for further exploration and development of plant-based health solutions.

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CHAPTER FIVE

5.0 GENERAL SUMMARY AND CONCLUSIONS

5.1 General Summary

This study explored the impact of various defatting methods on the physicochemical, functional, and bioactive properties of *Moringa stenopetala* (MS) seed protein isolates (MSPI) and their hydrolysates. Various defatting solvents (acetone, hexane, methanol, ethanol, water) were used and the defatted meal served as the raw material for two protein extraction methods: alkaline extraction followed by isoelectric pH precipitation and NaCl extraction combined with membrane ultrafiltration. The findings highlight significant advancements in enhancing the potential of MS proteins for food and nutraceutical applications.

Key results showed that the type of defatting solvent greatly affected the solubility, emulsifying properties, and thermal stability of the isolated proteins. The protein isolates from acetone-defatted MS meal (AMGPI) demonstrated superior overall functionality, with improved solubility, emulsification, and foaming properties. Protein isolates from methanol (MMGPI) and 70% ethanol (7EMGPI)-defatted MS meal also performed well, with 7EMGPI exhibiting strong stabilization of emulsions and foams. Solubility was highest at pH 3, although the acidic nature could limit application of the protein isolates in emulsion-based systems. In contrast, neutral and alkaline pH levels (7 and 9) proved more effective environments for emulsion and foam formation, offering better stability and air incorporation. Additionally, Mem_NaCl and Iso extraction methods demonstrated differing efficiencies in protein recovery and concentration. The Iso extraction method yielded higher protein content and yield compared to the Mem_NaCl method highlighting its effectiveness in concentrating proteins. This makes Iso extraction a promising technique for

isolating MS proteins for applications in nutraceuticals and functional foods. Enzymatic hydrolysis of the protein isolates produced protein hydrolysates with potential beneficial effects on human health. Hydrolysates generated with Alcalase (ALH) exhibited potent in vitro antihypertensive effects through strong inhibition of ACE and renin activities. Pepsin+pancreatin (PPH) hydrolysates were notable for their high protein recovery and antioxidant activity. The study also identified a link between the amino acid composition of MS seed meal-derived peptides and their functional health benefits, suggesting that bioactivity may depend on peptide structure rather than collective properties.

In conclusion, the research underscores the importance of defatting methods, particularly non-polar solvents like acetone, in maintaining protein functionality. Additionally, enzymatic hydrolysis was shown to enhance the bioactive potential of MS seed meal proteins, supporting their development as valuable components in nutraceuticals and functional foods.

5.2 Significance of Research

This research is significant for its dual focus on optimizing protein isolate production and harnessing bioactive peptides from MS. By examining the effects of defatting solvents on structural integrity and functional properties, the study provides a pathway for developing high-quality protein isolates tailored for food and nutraceutical applications. Furthermore, it highlights the therapeutic potential of bioactive peptides generated through enzymatic hydrolysis, showcasing their antihypertensive, antioxidant, and antidiabetic properties. These findings establish MS seed meal proteins and peptides as valuable components in functional foods and nutraceuticals. As consumer interest in natural, health-enhancing products grows, this research advances the understanding of MS seed meal peptides' bioactivity while promoting sustainable,

cost-effective solutions for improving human health and meeting the global demand for plant-based innovations.

5.3 Future Directions

Future research could further explore optimizing defatting methods to preserve the nutritional quality of MS proteins while enhancing functional properties. Investigating the synergistic effects of different enzymatic treatments on MS protein hydrolysates could lead to more tailored bioactive peptides with specific health benefits. Additionally, in vivo studies could validate the therapeutic potential of MS seed meal protein hydrolysates, particularly their antihypertensive, antioxidant, and antidiabetic effects. Another potential avenue for research is the application of MS protein isolates in various food formulations, especially those targeting functional beverages, emulsions, and foams, to evaluate their commercial viability. furthermore, future studies could focus on enhancing the taste profile of Moringa protein isolates, particularly through bitterness reduction strategies, to improve their acceptability in food products. Finally, expanding the scope of this study to include the impact of MS seed meal protein isolates on gut microbiota could further elucidate their role in human health, paving the way for plant-based solutions in functional food and nutraceutical development.