YELLOW FIELD PEA AND HEMP PROTEIN HYDROLYSATES AS TOOLS TO ENHANCE THE HEALTH BENEFITS AND SENSORY QUALITY OF MEAT ALTERNATIVES

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ABSTRACT

Protein hydrolysates are known to exert positive impact on human health and are useful in managing negative health conditions. This study produced enzymatic protein hydrolysates from hemp and pea, using papain and chymotrypsin, followed by incorporation into soybean-based meat analogues (MAs). The hydrolysates, hemp papain (HP), hemp chymotrypsin (HC), pea papain (PP) and pea chymotrypsin (PC) were used at the ratio of 8, 16 and 24% (w/w) of the soy protein concentrate (SPC) to develop MAs. The extruded MAs were tested for antioxidant, textural, bitterness intensity and in vitro bioactive properties. All the MAs had DPPH and metal chelating (MC) activities higher than their individual hydrolysates. The MC activities of the MAs were reduced with increasing hydrolysate content. SPC+8%HC and SPC+8%PP had the highest MC activity (42.99%) and were not significantly different from each other. However, all the hydrolysates had higher superoxide activities than their MAs, with the PP having the highest activity (35.43%) while SPC+16%HP had the highest hydroxyl radical scavenging activity (52.64 %). There were no significant differences in the α-amylase and α-glucosidase activities of the pea hydrolysates and their respective MAs, but the hemp hydrolysates had higher activities than their MAs. The hydrolysates had lower ACE inhibitory activities when compared to their MAs with SPC+8%HP having the highest inhibition (84.14%). The MAs had decreased hardness as the hydrolysates content increased while bitterness intensity was greater for the hydrolysates. The MAs that contained protein hydrolysates showed different variabilities in their cutting forces in both longitudinal and transverse directions, however, they all exhibited good cutting forces relative to the MA that contained only SPC. All the MAs had higher acetylcholinesterase (AChE) inhibitory activity than galantamine (AChE inhibitor drug), however, those made with pea protein hydrolysates showed better inhibition than the MAs that contained hemp hydrolysates. Results

from this study indicate the potential use of plant-based protein hydrolysates to enhance the sensory and health-promoting properties of MAs.

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LIST OF ABBREVIATIONS

MAs – Meat analogues

HP – Hemp papain hydrolysate

HC – Hemp chymotrypsin hydrolysate

PP – Pea papain hydrolysate

PC – Pea chymotrypsin hydrolysate

SPC – Soy protein concentrate

MCA – Metal chelating activity

ACE – Angiotensin-I converting enzyme

AChE – Acetylcholinesterase

DPPH - Diphenyl-1-picrylhydrazyl

FRAP – Ferric reducing antioxidant power

WHO - World Health Organization

NCD - non-communicable diseases

HWG – Hydrolyzed wheat gluten

ABTS – 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid)

ACE2 - Angiotensin converting enzyme 2

SHR - Spontaneously hypertensive rats

ROS - Reactive oxygen species

RNS - Reactive nitrogen species

HTST - High temperature short time

Wb – Wet basis

w/v – Weight per volume

HCl – Hydrochloric acid

DH – Degree of hydrolysis

DDQ - Double distilled water

BSA - Bovine serum albumin

ATCI - Acetylcholine iodide

DTNB - 5,5-dithiobis-(2-nitrobenzoic acid)

FAPGG – N-[3-(2-Furyl) acryloyl]-L-phenylalanylglycylglycine

DNSA - Dinitrosalicylic acid

4-MU - 4-methylumbellifer-one

4-MUoleate - 4-methylumbelliferyl oleate

GSH – Glutathione

EDTA – Ethylenediaminetetraacetic acid

MC – Moisture content

d.b – Dry basis

SME – Specific mechanical energy

TPA - Texture Profile Analysis

L* - Lightness

a* - Greenness-redness

b* - Blueness-yellowness

BCAA - Branched-chain amino acids

HAA – Hydrophobic amino acids

NCAA – Negatively charged amino acids

EAA – Essential amino acids

PCAA - Positively charged amino acids

DRSA - DPPH radical scavenging activity

ACh – Acetylcholine

F_L – Longitudinal

F_T - Transverse

AA – Amino acids

AAA – Aromatic amino acids

NaOH – Sodium hydroxide

SCAA – Sulfur-containing amino acids

SPC+8%HP - Soy-protein-concentrate meat analogue with 8% hemp papain hydrolysate

SPC+16%HP - Soy-protein-concentrate meat analogue with 16% hemp papain hydrolysate

SPC+24%HP - Soy-protein-concentrate meat analogue with 24% hemp papain hydrolysate

SPC+8%HC - Soy-protein-concentrate meat analogue with 8% hemp chymotrypsin hydrolysate

SPC+16%HC - Soy-protein-concentrate meat analogue with 16% hemp chymotrypsin hydrolysate

SPC+24%HC - Soy-protein-concentrate meat analogue with 24% hemp chymotrypsin hydrolysate

SPC+8%PP - Soy-protein-concentrate meat analogue with 8% pea papain hydrolysate

SPC+16%PP - Soy-protein-concentrate meat analogue with 16% pea papain hydrolysate

SPC+24%PP - Soy-protein-concentrate meat analogue with 24% pea papain hydrolysate

SPC+8%PC - Soy-protein-concentrate meat analogue with 8% Pea chymotrypsin hydrolysate

SPC+16%PC - Soy-protein-concentrate meat analogue with 16% pea chymotrypsin hydrolysate

SPC+24%PC - Soy-protein-concentrate meat analogue with 24% pea chymotrypsin hydrolysate

CHAPTER ONE

1.0 INTRODUCTION

The World Health Organization (WHO) reported that 36 million people die yearly through non-communicable diseases (NCDs) such as hypertension, diabetes, cardiovascular, and cancers (WHO, 2011). One of the four primary behavioral characteristics that contribute to the development and progression of NCDs is the consumption of unhealthy foods, which can be prevented and managed through nutritious foods and regular physical exercise (WHO, 2011). With respect to this, functional foods and nutraceuticals have emerged as alternative approaches to drug treatments, particularly in managing and preventing human diseases and maintaining ideal health conditions (Udenigwe & Aluko, 2012). As part of this approach, enzymatic digestion of food proteins into peptides has been used to produce ingredients suitable for the formulation of functional foods and nutraceuticals. In the last two decades, there has been increased attention and scientific research on food protein-derived bioactive peptides and hydrolysates, which show a wide range of health-promoting properties (Udenigwe & Aluko, 2012). In their native forms, food proteins do not reveal their biological functions, however, the intrinsic biological activities in the form of amino acid sequences can be released by chemical, enzymatic, and microbial hydrolysis (Wu & Chen, 2022). Among all the food sources, plants, particularly legumes such as lupin, peas, soy, and fava bean have been established as abundant sources of protein-derived peptides due to their high protein contents (Peighambardoust *et al.,* 2021).

Legumes supply about 33% of dietary plant proteins to human beings and are an important economic crop globally. They contain 16 – 50% proteins, bioactive ingredients, minerals, vitamins, and natural antioxidants (Bessada *et al.,* 2019). The nutritional quality of legumes is associated with their high-quality proteins, peptides, and balanced essential amino acid content (Bessada *et*

al., 2019). Consumption of legumes has been reported to reduce the risk of chronic diseases (Bessada *et al.,* 2019). Likewise, several studies have reported the ability of protein-derived bioactive peptides to manage hypertension, diabetes, thrombosis, microbial infections, and oxidative stress (Aluko, 2021; Mireku-Gyimah, 2021; Peighambardoust *et al.,* 2021).

Field peas have become popular over the years because of their low cost of production, health benefits and minimal negative environmental impact (Choudhury *et al.,* 2020; Barac *et al.*, 2010). Processing field peas into proteins, starch, and fiber-rich fractions can improve their value, and produce ingredients useful in formulating foods to enrich nutritional and techno-functional properties (Asen *et al.,* 2023). Pea proteins have a good amino acid profile when compared with other plant proteins such as soybeans and pulses, making them an ingredient with high potential for use in the food industry (Gorissen *et al.,* 2018). Yellow field pea seeds contain 21 – 22% protein content, making them suitable for producing isolated protein products (Millar *et al.,* 2019). Moreover, composite flours have been formulated using concentrates, isolates, and flours from pea proteins in combination with cereal and other legume ingredients to produce extruded food snacks (Banki *et al.,* 2021; Webb *et al.,* 2020; Chan *et al.,* 2019: Jebalia *et al.,* 2019). This fortification improved the nutritional quality of extruded snacks when compared to those extruded from cereals (Ge *et al.,* 2020). This information, combined with the reported bioactive properties of pea protein-derived peptides, suggests that yellow field pea hydrolysates could improve the nutritional composition of formulated food products and be useful in developing functional foods.

Hemp seed is mainly processed through mechanical pressing to extract the oil and the residue are used in making numerous protein-rich food products. Hemp seed contains approximately 25% protein content (House, 2010), with 75% salt-soluble (globulins), and 25% water-soluble (albumin) as the main storage proteins (Aluko, 2017). Hemp seed proteins are rich

in sulfur-containing amino acids and arginine, which contribute to their high nutritional value (Aluko, 2017). Moreover, enzymatically hydrolyzed hemp seed hydrolysates are effective against oxidative stress and hypertension through *in vitro* and *in vivo* tests (Malomo *et al.,* 2015). However, the use of protein hydrolysates in food product development has not been well explored, particularly in high moisture extrusion cooking. Therefore, this research aims to incorporate plant-derived protein hydrolysates from hemp, and yellow field pea into formulated plant-based meat analogues.

Meat analogues are meat replicas made from alternative sources such as plants. They possess equivalent properties to animal meat in terms of texture, taste, appearance, and flavor (Zhou *et al.,* 2021). Adding protein hydrolysates to produce meat analogues could be an excellent means to introduce these hydrolysates to consumers and improve the nutritional composition of meat analogues, and possibly the sensory properties. This will add value and increase diversification in the use of agricultural produce and its basic nutritional functions, particularly as sources of active ingredients for formulating health-promoting food products.

In a work conducted by Zhang *et al.* (2023), meat analogues were produced using soybean protein concentrate with hydrolyzed wheat gluten (HWG) at different ratios. The addition of HWG improved the degree of fibrous structure formation of meat analogues. As the concentration of the HWG increased, the fibrousness degree of the meat analogues also increased. The authors attributed this trend to the interactions between the soybean protein and the outer cysteine residue peptide chains of HWG, forming a strong fibrous network structure (Tuck *et al.,* 2014). However, the authors noted that the fibrousness degree started decreasing at some levels of HWG (above 40%), meaning the addition of HWG has an optimum level. As the level of HWG increased, the hardness, toughness, and chewiness decreased. The meat analogues containing 40% of HWG had

the lowest hardness, toughness, and chewiness values, similar to values obtained for chicken (Grabowska *et al.,* 2014). This suggests that adding hydrolysates to meat analogues produces a softer and easier to chew meat analogue while maintaining a similar fibre level as chicken muscle. Adding HWG to soybean protein concentrate also significantly improved the meat analogues' antioxidant capacity, which was tested from DPPH and ABTS radical scavenging with higher antioxidant capacity at higher HWG concentrations (Zhang *et al.,* 2023). In a similar study by Razavizadeh *et al.* (2022), adding 10% hydrolysates from soybean press cake resulted in no significant difference in the hardness and chewiness of the meat analogues and the control. Likewise, no significant difference was observed in the acceptability and sensory quality of the meat analogues produced with hydrolyzed soybean press cakes and the control sample.

1.1 Hypothesis

The inclusion of hemp or pea protein hydrolysates in soybean-based plant protein formulations will improve the textural quality, as well as the nutritional and health benefits of meat analogues.

1.2 Objectives

1.2.1 General objective

The overall objective of this study is to optimize extrusion cooking processing to manufacture soybean protein-based meat analogues that contain protein hydrolysates.

1.2.2 Specific objectives

a. Enzymatically hydrolyze hemp, and yellow field pea proteins and determine their in vitro bioactive properties such as antioxidant, anti-diabetic, anti-hypertensive and anti-neurodegenerative.

b. Incorporate each protein hydrolysate into soybean-based meat analogues developed through extrusion cooking.

c. Determine the effect of protein hydrolysates on the textural and taste properties of formulated meat analogues.

d. Compare the bioactive properties of hemp and yellow field pea hydrolysates with those of the formulated meat analogues.

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CHAPTER TWO

2. LITERATURE REVIEW

2.1. Food-derived protein hydrolysates

Amino acids are linked together by peptide bonds to form bioactive peptides, which have been established to exert positive impact on human health and body functions (Sanchez & Vazquez, 2017). In living organisms, particularly human beings, bioactive peptides exhibit important roles in their metabolic functions (Sanchez & Vazquez, 2017). Recently, bioactive peptides have been identified from protein sources ranging from different food products such as vegetables, soybeans, cereals, potatoes, nuts, eggs, dairy products, and meat proteins (Peighambardoust *et al.,* 2021). These peptides are usually produced from their parent proteins, and they have been shown to possess more and better bioactivities, which are beneficial to human health when compared to whole proteins (Udenigwe & Aluko, 2012). Food proteins mostly do not reveal their actual biological activities in their native form. However, these biological activities present in the proteins can be released by hydrolysis; chemical, enzymatic, and microbial hydrolysis (Wu and Chen, 2022). Among these three types of hydrolysis, enzymatic is the most efficient and effective technique for extracting protein-derived bioactive peptides (Wu & Chen, 2022).

Peptides are usually obtained from fish, eggs, milk, and pig skin, which are animal sources, and plant sources such as soybean, and spinach (Peighambardoust *et al.,* 2021). Most studies on bioactive peptides have focused on using animal proteins, especially from milk (whey and casein), muscles of meat and eggs, as well as marine sources of protein such as seahorse, shrimp, squid, sea urchin, salmon, oyster, fish, macroalgae and snow crab (Udenigwe & Aluko, 2012). Pulses (pea, beans, lentil, and chickpea), flaxseed, hemp seed, wheat, oats, canola, soy are the common plant food proteins used in the production of bioactive peptides (Udenigwe & Aluko, 2012). There

are two major criteria for selecting food proteins as sources of bioactive peptides, which are (1) an attempt to add value to numerous underutilized proteins and protein-rich by-products from the food industry and (2) the use of proteins with amino acid residues containing pharmacological interest and particular peptide sequences (Udenigwe & Aluko, 2012). Peptides often exhibit superior bioactivity when compared to their parent proteins, highlighting the importance of hydrolysis in releasing bioactive peptides (Girgih et al., 2014). Enzymatic hydrolysis and fermentation are used to produce the most reported bioactive peptides. Enzymatic hydrolysis is done with the use of a single or multiple specified or non-specified proteases to produce the peptides of interest (Udenigwe & Aluko, 2012).

2.1.1 Plant-derived bioactive peptides

Various plant-derived peptides are available and extracted mostly using dicot plants in which the method of extraction is mainly enzymatic protein digestion. These peptides are similar in chemical composition to proteins; however, their molecular weight is lower than proteins, having about 10 kDa or less (Yuan et al., 2022). The structures of these peptides are made up of α-helices, β-sheets, linear and hybrid structures, which are mainly determined by the sequence of amino acids (Yuan et al., 2022). The sequence of the amino acids and the specialized structures impact biological functionality. Bioactive peptides are grouped into several types based on functionality and structural attributes. They are cyclic peptides, orbitides, glycinins, angiotensin-converting enzyme (ACE) inhibitory peptides, defensins, cyclotides, heveins, knottins, and many others (Yuan et al., 2022). They exhibit various functional properties due to the sequence of their amino acids and their structures. Peptides are presently available in the commercial market, sold as food and drink, powders, tablets, liquids, and capsules such as peptide soup, Evolus and Calpis (Lafarga et al., 2020).

2.2 Methods of protein hydrolysis

Plant-based protein hydrolysates can be produced from protein sources using different methods such as microbial fermentation using proteolytic bacteria, enzymatic hydrolysis using proteolytic enzymes and chemical hydrolysis (Saadi et al., 2015; Korhonen & Pihlanto, 2006). Various types of peptides are produced from protein sources such as plant proteins by hydrolyzing with proteases (Lee et al., 2017).

2.2.1 Microbial fermentation

Fermentation generally means a process of incubating certain types of microorganisms with organic materials (Raveschot et al., 2018). The microorganisms produce hydrolysates by secreting hydrolytic enzymes, which break down specific components of the starting materials and hence produce hydrolysates. The fermentation process is an environmentally safe, friendly and economical process for producing different bioactive peptides (Gao et al., 2021). Balakrishnan et al. (2011) produced hydrolysates using lactic acid bacteria to ferment tannery fleshings and this hydrolysate showed high antimicrobial and antioxidant activities. Jemil et al. (2014) used a proteolytic bacterium *Bacillus subtilis* to ferment various fish proteins into protein hydrolysates. All protein hydrolysates produced via fermentation displayed dose-dependent antioxidant and antimicrobial activities, which were evaluated through various *in vitro* assays. Fermentation of turbot skin using *Aspergillus oryzae* resulted in protein hydrolysates with high antioxidant activity (Fang et al., 2017). Several of the industrial microorganisms used in fermented dairy production are highly proteolytic, enabling them to generate bioactive peptides during the fermentation process (Castellano et al., 2013; Je et al., 2005). For example, the tripeptide sequences Val-Pro-Pro and Ile-Pro-Pro which were the first highly potent angiotensin-converting enzyme (ACE) inhibitors discovered in fermented milk (Sipola et al., 2002; Nakamura et al., 1995). Fermentation offers some advantage such that the hydrolysis is enzymatically driven by the microorganism's

proteases and therefore, the bioactive peptides are purified without any further hydrolysis. However, the process is disadvantageous because it produces low yield of peptides (Nasri, 2017). In the fermentation process, some of the bioactive peptides and amino acids produced by proteolytic enzymes from food proteins are used by the microorganisms as nitrogen and or carbon substrate for their growth (Nasri, 2017).

2.2.2 Chemical Hydrolysis

Protein hydrolysates are produced via chemical hydrolysis by using either acid or alkali solutions to break the peptide bonds (Nasri, 2017). This method is advantageous because it is relatively simple and cheap (Gao et al., 2021). However, chemical methods with the use of strong acids or bases are unacceptable ecologically and the molecular weight of peptides generated from this method is difficult to control (Nasri, 2017). Also, chemical methods often cause unwanted side reactions, including degradation of tryptophan and racemization, and may also result in the formation of significant salt products with less nutritional value and biological functions (Gu´erard & Sumaya-Martinez, 2003). Acid hydrolysis often causes oxidation of methionine and cysteine, degradation of some threonine and serine, and occasionally change asparagine and glutamine to aspartate and glutamate (Bucci & Unlu, 2000). Chemical hydrolysis makes it impossible to reproduce the bioactivities because there is no specificity in the cleavage of peptide bonds by chemical reagents, therefore the variability in hydrolysis causes large variation in the bioactivity (Nasri, 2017). These drawbacks have greatly restricted the high-value applications of these protein hydrolysates from chemical hydrolysis.

2.2.3 Enzymatic hydrolysis

Enzyme-based protein digestion is the most common method of releasing bioactive peptides from their parent protein. In enzymatic hydrolysis, the peptide bonds formed in the parent

protein are broken by the action of commercial enzymes, which release the encrypted peptides to produce bioactive peptides (Udenigwe & Aluko, 2012). Enzymatic hydrolysis has been carried out in three ways (1) conventional batch methods, (2) immobilized enzymes, (3) and lastly ultrafiltration membranes (Cruz-Casas et al., 2021). Pretreating parent proteins has been used to improve enzymatic hydrolysis, and this resulted in better rates of hydrolysis with minimal quantity of enzymes. Moosavi et al. (2020) have used different protein pretreatment methods such as ultrasound, hydrostatic high-pressure assisted enzymatic hydrolysis, microwave, and high voltage pulsed electric field. Proteases and carbohydrases cleave proteins and break down the cell wall, respectively to release peptides. The primary principles involve selection of the suitable enzyme based on properties of the parent proteins and conduct of protein hydrolysis under appropriate conditions to produce the peptides (Cruz-Casas et al., 2021). Papain, pepsin, trypsin, and proteases (neutrase, alcalase, flavourzyme, and protamex) have all been used in the extraction of peptides (Tang et al., 2009). Montone et al. (2018) extracted angiotensin converting enzymes (ACE) inhibitory peptides from the by-products of cauliflower (stems and leaves) with the use of shotgun peptidomics and alcalase. Enzymes also act as catalysts by speeding up the reaction to break down the proteins, and the speed is generally higher with about 10^6 to 10^{12} times than the un-catalyzed extraction (Martínez-Medina et al., 2019).

Enzymatic hydrolysis usually takes place at a mild temperature and pH values, with temperatures of <100 °C, and pH <10, which contributes to cost and energy savings (Martínez-Medina et al., 2019). In addition to the higher yields from enzymatic hydrolysis, the process is quite simple, results in superior quality, and the enzyme inactivation is readily achieved. The properties of protein hydrolysates, including amino acid composition, and the length and molecular weight of peptides can be affected by numerous factors, which include pH, time of hydrolysis,

substrate, enzyme ratio and the temperature at which the reaction takes place (Li-Chan, 2015). All these factors must be controlled and monitored to be in their optimum condition because they can affect bioactivity of the generated protein hydrolysate. Enzymes are specific in their actions and hence it is important to consider their specificity because the type of enzyme used for hydrolysis influences the bioactive potency of the peptides produced. This was seen in the study of Ferri et al. (2017), who hydrolyzed rice proteins using bacillolysin and found out that the peptides demonstrated improved anti-tyrosinase and anti-inflammatory activities as compared to peptides from papain or subtilisin hydrolysis. In another study, dipeptides containing tyrosine and tryptophan, hydrophobic peptides and ACE inhibitory short-chain peptides were produced by enzymatically hydrolyzing pea, rice, soy, and wheat protein using thermolysin and chymotrypsin after which butanol extraction was done (Rudolph et al., 2017).

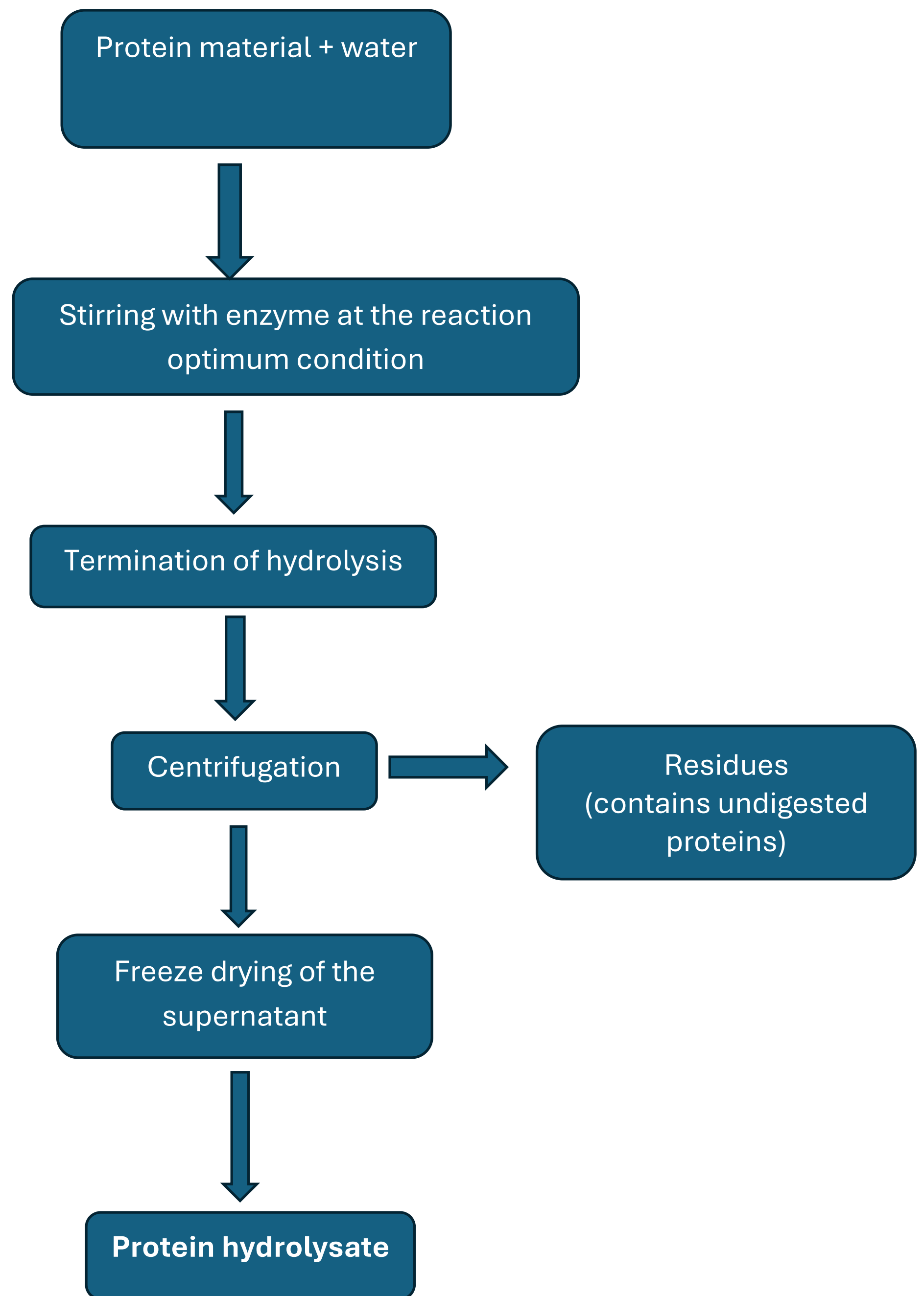

Figure 1: Flowchart of enzymatic hydrolysis process to produce protein hydrolysates.

Developed using the method adapted from Udenigwe and Aluko (2012).

Table 1: Advantages and disadvantages of various peptide preparation methods

Methods of Peptide Preparation	Advantages	Disadvantages
Chemical extraction	1. Technologically mature 2. Application ready	1. Chemical reagents 2. Equipment cost 3. Environmental pollution 4. Lack of product reproducibility 5. Production of toxic compounds 6. Destruction of amino acids
Fermentation	1. Good taste 2. High yield 3. Low cost	1. Long fermentation time 2. Safety problems
Synthesis	1. Technologically mature 2. High purity	1. High cost 2. Low yields 3. Presence of residual chemicals
Enzymatic hydrolysis	1. Easy process control 2. Low energy consumption 3. High repeatability and reproducibility 4. Low cost	1. Low efficiency 2. Long enzymolysis time

Source: Wen et al. (2020)

2.3 Yellow field pea seed and its protein hydrolysates

Field peas, also known as yellow peas, are the dried seeds of *Pisum sativum L* and are an important pulse crop with several nutritional and agricultural benefits. Yellow field pea is one of the most grown legumes globally and its proteins are nutritionally beneficial (Choudhury et al., 2020). They are grown globally, with Russia, China, and Canada being major producers, and Canada being the leading exporter (Statistics Canada, 2022). Their high seed protein content (22-25%) makes them a valuable source of plant-based protein. While pea protein is considered a complete protein (containing all nine essential amino acids), it is not balanced because of the limited content of methionine. In addition to protein, peas also provide complex carbohydrates, fibre, vitamins, and minerals, which contribute to various health benefits (Pelgrom et al., 2013).

Pea seeds generally contain low fat, rich in protein, starch, and micronutrients including minerals and vitamins (Choudhury et al., 2020). Field peas are becoming more popular daily because of the numerous beneficial health effects, low cost of production, and because they are environmentally sustainable (Choudhury et al., 2020). Processing raw field peas into proteins, starch, and fractions rich in fibre, enhances the nutritional and economic values of the peas. These components are used for formulating nutritionally enriched and technologically superior food products (Gorissen et al., 2018). The quantity and quality of pea protein determines its nutritional composition and functional properties (Gorissen et al., 2018). When compared with other proteins from plant sources such as soybeans, chickpeas, and beans, pea protein has a better amino acid profile, hence it has a high potential as an ingredient in the food industry (Gorissen et al., 2018). In contrast to the whole protein, pea hydrolysates have not been well explored in food product development.

Pea protein hydrolysates have been shown to possess various bioactivities, including antihypertensive, anti-adhesive and anti-inflammatory effects (Ndiaye et al., 2012; Li et al., 2011). Awosika & Aluko (2019) extracted hydrolysates from yellow field pea protein after chymotrypsin, trypsin, pepsin or alcalase hydrolysis and examined *in vitro* activities with respect to inhibitions of α-glucosidase, α-amylase, and pancreatic lipase. From this study, pea hydrolysates and their peptide fractions inhibited α-amylase, pancreatic lipase, and α-glucosidase. The activity of the hydrolysates and peptide fractions increased as the peptide concentration increased. For the α-amylase, at the highest concentration of 225 μg/mL, among all the samples, the chymotrypsin fraction 1-3 kDa inhibited the most, with the value of 30.52 ± 0.01%. In another study by Liao et al. (2019), protein hydrolysates were produced from yellow pea and peptides that exhibited angiotensin converting enzyme 2 (ACE2) upregulating activities were discovered. The

identification of ACE2 upregulating peptides from pea protein hydrolysate further proves that pea protein is a valuable source for discovering a wide spectrum of bioactive peptides.

2.4 Hemp seed and its protein hydrolysates

Industrial hemp seed is continually becoming an important crop in Canada. Hemp seed is rich in protein (25%), has approximately 35.5% oil (mainly polyunsaturated fatty acids), 20 - 30% carbohydrates, 5.6% ash, and 28% total fiber (5.4% digestible and 22.2% non-digestible) (Callaway, 2004). Hemp seed is also rich in vitamins and minerals, and contains lesser amounts of antinutritional factors (tannins, phytic acid, trypsin inhibitor and tannins) when compared to rapeseed and soybean (Leonard et al., 2020). Hemp is rich in fibre and the vegetative part has been used widely for the production of clothing and paper in Canada (Lu et al., 2010). The key hemp products in the health food market include whole hemp seeds, hulled hemp seeds, hemp seed oil, and hemp protein meal. These hemp-based foods contain minimal tetrahydrocannabinol levels, making them non-intoxicating when consumed (Karimi & Hayatghaibi, 2005). Among plant proteins, hemp seed proteins contain a significant level of arginine, accounting for about 11% of the protein weight, while other protein sources are mostly < 6%. The health-promoting bioactive compounds present in hemp seed proteins have enhanced their value-added use in functional foods and nutraceuticals, leading to a rise in hemp seed products on the market and increasing the crop's economic value (Girgih et al., 2014).

Proteins and peptides from hempseed have continually received attention over the past decades because of their high protein content and beneficial properties. This interest is because of the increase in research into the health benefits of proteins and hydrolysates produced from vegetables. So far, among plants used in bioactive peptides production, hemp seed stands alongside canola, pulses, soybean, flaxseed, oat, and wheat (Santos-Sánchez et al., 2022). Many researchers

have produced bioactive peptides having desirable health benefits from hemp seed, some of which are protective agents against oxidative apoptosis (Chakrabarti et al., 2014), while others exhibited antioxidant (Girgih et al., 2014a), and antihypertensive (Girgih et al., 2014b) properties. Hemp seed protein hydrolysates have also been evaluated for basic industrial or techno-functional properties (Teh et al., 2014). Tang et al. (2009) studied the antioxidant properties of hempseed hydrolysates in cell-free systems and found them exhibiting high DPPH scavenging and Fe^{2++} chelating properties. Girgih et al. (2011) reported the ability of hemp seed protein hydrolysates produced with pepsin and pancreatin digestion to inhibit ACE activity in an *in vivo* study where 200 mg/kg (body weight) was orally administered to spontaneously hypertensive rats (SHR) and the systolic blood pressure measured over 24 h. The hydrolysate demonstrated an effective activity against hypertension in the SHR with a 30 mmHg decrease in systolic blood pressure after 8 h, which is comparable to the effectiveness of captopril, the antihypertensive drug (3 mg/kg body weight). In a subsequent study, Malomo et al. (2015) also demonstrated similar *in vivo* activity after oral administration of hemp protein hydrolysate (200 mg/kg body weight) obtained from papain, pepsin, or alcalase. It was established that hempseed hydrolysates had a higher ACE inhibitory activity than other hydrolysates produced from almond, sweet potato, and pistachio. Hence, the results demonstrated the significance of types of food proteins, enzymes, and hydrolysis conditions in defining the biological activity of protein hydrolysates.

2.5 Food Protein-Derived Bioactive Peptides and Human Health

Bioactive peptides in humans are mostly involved in repairing and maintaining the body, as well as participation in biochemical reactions and energy synthesis (Ndiaye et al., 2012). The bioactivities of these peptides have been explored well in recent years. For instance, plant proteins from food sources such as rice, wheat, and vegetables, when consumed directly produced peptides

in the body (Fan et al., 2022). The biological activities of these peptides depend largely on the amino acid composition of the peptides. These amino acid sequences are inactive when encoded in the animal and plant protein structures but become active when released during different processes such as *in vitro* or *in vivo* enzymatic hydrolysis, fermentation, and food processing (Aluko, 2008; Hartmann & Meisel, 2007). Although the activities of synthetic drugs are more potent than peptides in targeting diseases, however, bioactive peptides have numerous advantages over synthetic drugs in terms of disease intervention, which includes reduced healthcare costs, and safety of natural products. Protein hydrolysates also serve as an extra source of essential amino acids, which are beneficial to vulnerable people who require special health care such as the aged people and pregnant women (Girgih et al. 2014). Bioactive peptides can in different ways have effects on the body's four major systems when administered orally. This includes: (1) the digestive system, by functioning as antimicrobial agents, opioid peptides, mineral-binding compounds and appetite suppressants; (2) opioid activity in the nervous system; (3) the cardiovascular system where bioactive peptides function as antihypertensive, antioxidants, antithrombotic, hypocholesterolemic and antilipidemic agents; and lastly (4) in the immune system, bioactive peptides exhibit cytomodulatory, immunomodulatory, opioid and antimicrobial properties (Korhonen and Pihlanto, 2006).

The peptides released through hydrolysis generally exhibit stronger bioactivities than their intact parent proteins, indicating that breaking the peptide bonds is important to enhance their potency. Consequently, the ability of specific sequences of dietary peptides to lower the risk of chronic diseases, enhance human health and strengthen natural immune defenses has attracted attention from scientists and even in the commercial industries for decades (Hartmann & Meisel, 2007). Peptides show diverse biological activities and can exert beneficial effects on human health

through various means. Plant-derived protein hydrolysates demonstrated effective biological activities which include antioxidant, anticancer, lipid-lowering properties, antimicrobial, antihypertensive and immunomodulatory, which is mainly because of their component peptides (Udenigwe & Aluko, 2012). The structural attributes are the primary determinants of their specific bioactivity towards these molecular diseases, which include length of their chains and the physicochemical properties of the amino acid residues, such as, molecular charge, hydrophobicity, and side-chain bulkiness (Ndiaye et al., 2012). These hydrolysates have potential in food product development to positively improve human health such as in chemoprevention (Ndiaye et al., 2012).

2.5.1 Antioxidant effects

Antioxidants are substances which have the capacity to alleviate oxidative stress in the body by chelating metal ions, scavenging free radicals and preventing, delaying or inhibiting oxidation of nutrients and cellular components (Girgih et al., 2014). The reactive oxygen species (ROS) or reactive nitrogen species (RNS), which include hydrogen peroxide, peroxynitrite, superoxide anion and hydroxyl radical when in excess can suppress the activities of natural antioxidants in the body. This imbalance can cause damage to biomolecules like proteins, enzymes, lipids and DNA (Girgih et al., 2014). Degenerative diseases, such as coronary heart disease, cancer, atherosclerosis, Alzheimer's disease, diabetes, and hypertension, are partially attributed to an increased production of free radicals in the body. These free radicals can lead to DNA mutations, protein malformations, and phospholipid oxidation (Diaz et al., 1997; Halliwell, 1994).

Peptides have the capacity of chelating metal ions, scavenge free radicals and serve as protective barriers (Manzoor et al., 2022). The mechanism of scavenging radicals is through electron donation as well as maintaining stability based on structural resonance. Also, peptides possessing amino groups and carboxyl on their side chains chelate metal ions because they can

separate out and donate electrons and hydrogen atom and therefore prevent the formation of free radicals by stabilizing metal-based pro-oxidants (Girgih et al., 2014). Peptides may act as physical barriers or membranes, protecting against lipid peroxidation due to their surfactant qualities, which limit the interaction between lipids, radicals, and other oxidizing agents (López-García et al., 2022). Hydrolysates of different food proteins possess antioxidant activities as discovered from *in vitro* and animal models including camel milk casein (Kumar et al., 2016), *Sphyrna lewini* muscle (Luo et al., 2013), whey protein (Peng et al., 2009), and sardinelle (Bougatef et al., 2010). The total antioxidant activity of protein hydrolysates is largely dependent on the combined attributes of multiple mechanisms such as free radical scavenging, scavenging of compounds containing oxygen, metal ion chelating, inhibition of lipid peroxidation and reducing capacity (singlet electron transferring) (Chen et al., 1998).

This is primarily due to the various antioxidant activities of bioactive peptides. Many factors affect the antioxidant activity of peptides, which include constituents of the hydrolyzed substrate, processing conditions (substrate/enzyme ratio, pH, and temperature), the enzyme of choice and also the degree of hydrolysis (Laroque et al., 2008). Laroque et al. (2008) noted that hydrolyzing food proteins for 4 h resulted in the best DPPH radical scavenging by mackerel hydrolysates. Antioxidants can perform their activities using different systems and it is recommended to assess antioxidant activity using different types of assays (Frankel & Meyer, 2000).

2.5.2 Anti-obesity and anti-inflammatory effects

There has been extensive study of hemp seed protein hydrolysates and some selected hemp seed peptides against inflammatory diseases. Rodriguez et al. (2019) showed that addition of hemp seed hydrolysates produced from alcalase alone or a combination of flavourzyme and alcalase to

lipopolysaccharide stimulated microglia cells of BV-2 led to reduced levels of proinflammatory cytokines, including interleukin-6, interleukin-1β and tumor necrosis factor, while the mRNA levels of the anti-inflammatory cytokine interleukin-10 were increased. The authors attributed the activity of hemp seed hydrolysates in protecting against neurodegenerative diseases to their potent antioxidant capacity as established in previous analysis. This association is significant because of the observed relationship between oxidative stress and chronic inflammation, particularly in neurodegenerative diseases like Parkinson's and Alzheimer's.

Pancreatic lipase is an enzyme involved in the digestion of dietary triacylglycerols in the intestine, which is a key source of excess calorie consumption (Chia et al., 2023). By inhibiting the activities of pancreatic lipase, fat absorption efficiency in the small intestines can be controlled to reduce body weight (Chia et al., 2023). Therefore, the reduction in the rate of absorption of fats such as triacylglycerols by inhibiting pancreatic lipase activity has given good directions to treat obesity (Chia et al., 2023). Awosika and Aluko (2019) produced peptides from yellow field pea using chymotrypsin, alcalase or pepsin hydrolysis and determined their ability to inhibit pancreatic lipase activity. This experiment showed more than 50% reduction in the activity of pancreatic lipase in the presence of the pea peptides. Effectiveness of the enzyme inhibition was measured by calculating the IC_{50} values whereby lower values indicate stronger potency as enzyme inhibitor. Samples hydrolyzed with trypsin (IC_{50} = 3.95 ± 0.04 mg/mL) and alcalase (IC_{50} = 3.98 ± 0.4 mg/mL) gave the lowest values, which shows better potency against pancreatic lipase enzyme than the pepsin and chymotrypsin hydrolysates. These results show the differences in effectiveness of proteases in producing hydrolysates that inhibit enzyme activity.

2.5.3 Antihypertensive effects

Several plant-derived bioactive peptides have been proven to inhibit the activities of ACE and renin, the most important enzymes that control mammalian blood pressure. Most experimental studies evaluating the potential of these peptides to prevent or treat hypertension have been conducted *in vitro*, but the outcomes do not always correspond to effectiveness *in vivo* (Hernández-Ledesma et al., 2011; Jauhiainen & Korpela, 2007). These differences in the *in vitro* and *in vivo* studies of antihypertensive activity could be because of degradation and functional inactivation of peptides by digestive enzyme during gastrointestinal transit (Girgih et al., 2014). Several studies have been carried out on hemp seed, and it has been established by multiple studies that different hemp seed protein hydrolysates exhibit ACE-inhibitory activity. Particularly, those obtained from different enzyme combinations such as papain, pepsin, protease, and alcalase have been proven to have the ability to reduce *in vitro* ACE activity (Malomo et al., 2015). Among the lists of hydrolysates particularly are those obtained by hydrolyzing using pepsin, alcalase, pancreatin, and a combination of pancreatin and pepsin, which were also established to inhibit renin activity (Girgih et al., 2011).

These experiments have proven that some specific categories of peptides are the most potent ACE inhibitors. Examples of such peptides include those that are relatively small in size, typically consisting of 2-20 amino acids. These peptides contain residues with cyclic or aromatic rings, like proline, phenylalanine, tyrosine, and tryptophan. Additionally, those that contain aliphatic chains like valine, glycine, leucine, and isoleucine positioned at the N-terminal end are also strong ACE inhibitors (Malomo et al., 2015). Several other studies have shown that hemp and pea seed protein hydrolysates can inhibit renin activity. The ability of a bioactive peptide to inhibit ACE is dependent on various factors such as maintaining peptide structural integrity and being active upon reaching target organs. Other factors include peptide resistance to digestion by

proteinases and peptidases, and successful transport across the brush border membrane while maintaining its structural integrity (Girgih et al., 2014). Girgih et al. (2011) conducted a study by replicating the human digestive system in a controlled laboratory to produce hemp seed protein hydrolysates. The authors showed that the protein hydrolysates from hemp seed inhibited activities of renin (IC_{50} of 0.81 mg/mL) and ACE (IC_{50} of 0.67 mg/mL) *in vitro*. The hemp seed protein hydrolysate showed better renin inhibitory activity than the membrane ultrafiltration fractionated peptides and the unhydrolyzed hemp seed protein.

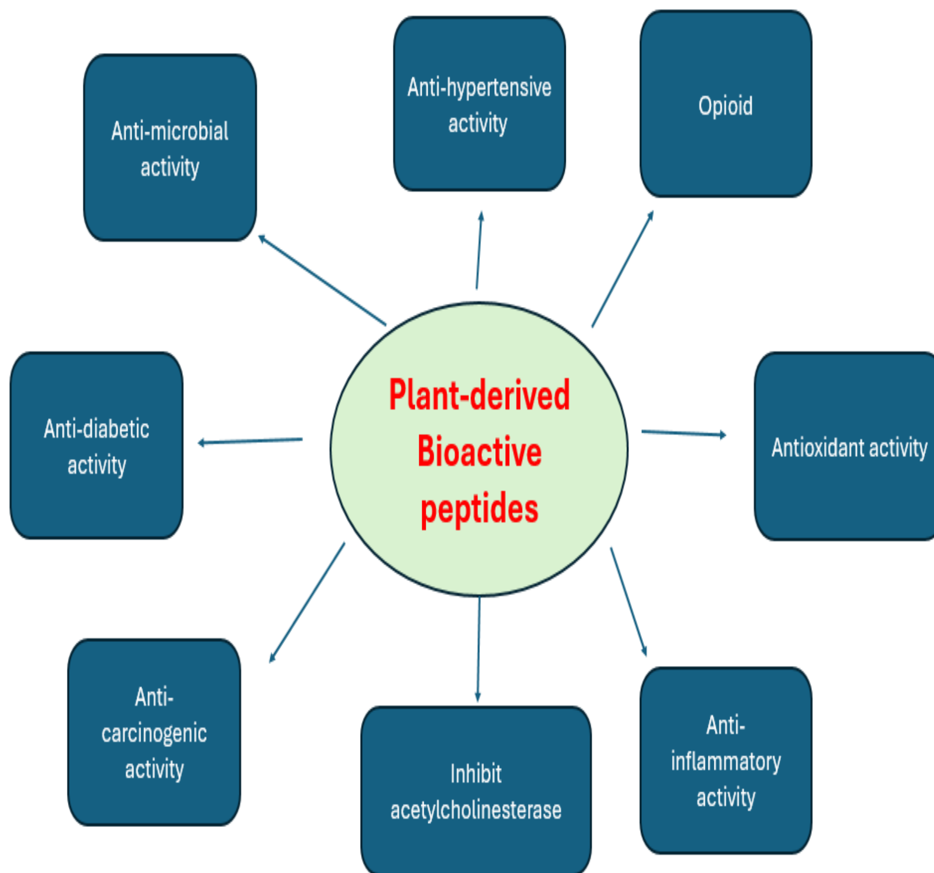

Figure 2: Some health benefits of bioactive peptides.

Table 2: Some bioactive peptides and their functionalities.

Plant-derived peptides	Amino acid sequence	Mechanism	Activity
Lupin	LILPKHSDAD	HMGCoAR activity↓, LDL uptake↑, PCSK9 and HNF1-alpha protein↓	Cholesterol-lowering activity
Yellow field pea	LTFPGFEGTVFENG IFENLQN FEGTVFENG	ACE and renin activity↓, systolic blood pressure↓	Antihypertensive activity
Hemp seed	WYT, SVYT, and IPAGV	Inhibit the catalytic activity of HMGCoAR and exert hypocholesterolemic effects with a statin-like mechanism	Hypocholesterolemic activity
Soybean	IY and WMY	Display ACE-inhibitory activity through forming a variety of H-bonds with the C-domain of ACE; show resistance to in vitro digestion by gastrointestinal proteases	Antihypertensive

Extracted from: Fan et al. (2022) and Zanoni et al. (2017).

2.6 Applications of plant-derived protein hydrolysates in food processing

The use of protein-based hydrolysates in food processing is taking the lead in the advancement of modern nutrition which offers an efficient way of delivering bioactive compounds (Singh et al., 2023). Functional foods and nutraceuticals fortified with protein hydrolysates and peptides are increasingly growing, especially for people who require specific nutritional needs such as elderly, infants, and athletes, because peptide digestion is relatively fast (Singh et al., 2023). This has led to many shelf-stable functional food products being developed with numerous health promoting benefits. This is mainly because these compounds are enzymatically hydrolyzed from

their parent proteins, which is more cost effective and the end products from this hydrolysis can bypass gastrointestinal tract digestion and thereby reaches the target molecule to perform their expected physiological functions (Girgih et al., 2014). Protein hydrolysates are becoming more appealing to consumers because of the absence of negative side effects, which make them relatively safe and unlike the pharmaceutical drugs, peptides have higher dosage limitations. As a result, some functional foods and nutraceutical products with various health claim statements on their labels have been approved and are now readily available in supermarkets and drugstores for consumers seeking various health benefits (Girgih et al., 2014). Furthermore, some bioactive peptides have been incorporated into the cosmetic industry to support skin health and address dermatological conditions (Girgih et al., 2014). The bioavailability and effectiveness of bioactive peptides in the body can be influenced by physiological changes in the gastrointestinal tract (Escudero et al., 2014). For these peptides to be successfully incorporated into the diet, their active sequences must withstand digestion. Only by maintaining their structure can they reach the intended body organs to exert their intended effects.

2.6.1 Extrusion cooking process

Different industries make use of extrusion technology including the food industries, packaging and plastic industries, ceramics and metal fabrication industries (Li, 2021; Wittek & Emin, 2017). Extrusion technology is a versatile technique, which was introduced back in the late 1930s for use in the pasta industry. This is a cold form of extrusion usually at temperatures below 100 °C (Fellows, 2017; Bouvier & Campanella, 2014a), while extrusion cooking was introduced in the late 1940s and was used to develop animal feed. However, in the 1960s, ready-to-eat breakfast cereals were the first human being foods, which were made using the extrusion process (Judson Harper, 2019). Extrusion cooking is a high temperature short time (HTST) process with

temperature reaching 170 °C and a residence time of 2-5 min (Cornet et al., 2022; Judson Harper, 2019). This is used in the food industries to produce several new products including breakfast cereals, baby foods, and modified starches from grains (Mościcki & van Zuilichem, 2011). The process comprises multiple unit operations including shearing, cutting, heating, shaping and mixing, which cause changes to the structure and the techno-functional properties of the food (Morales Alvarez, 2020). An extruder is made up of different important components, which include a motor that is responsible for rotating the shafts, a feeder for feeding raw ingredients into the extruder, a hopper for holding the raw material, a die for giving shape to the products, the cutter for cutting the finished products into the same length, the screw assembly housed in the barrel, a water injection port, and a barrel with multiple temperature zones.

Zhang et al. (2019) described a standard extrusion cooking process as where raw ingredients are passed through four different zones, starting from the feeding region to the mixing region, the melting region (where they are cooked), and to the die region. The feeding zone receives the raw ingredients and then passes them into the mixing region through the help of the rotating screws. Water flows into the extruder from an inlet in the mixing zone and then mixes with the incoming raw ingredients. The water flow rate is controlled to achieve the required moisture content in the product (Zhang et al., 2019). The melting region is one of the most important regions because this is where the raw ingredients are cooked by the high temperature in the barrel and the strong shear forces exerted on it by the screws. The material leaving this zone, and usually called the melt, proceeds to the die region, which gives it the desired shape. The final structure of the extrudate is set due to evaporation of water as a result of the sudden pressure drop (Zhang et al., 2019). As the extrudate exits the die, its final structure is established when water evaporates due to the drop in pressure. Depending on the expected final product such as meat

analogues or puffed snacks, the required extrusion parameters will vary. The parameters include ingredients type, screw configuration and speed, extruder barrel temperature, die type, as well as the dry and wet feed rates.

2.6.1.1 High moisture extrusion cooking for meat analogues

High moisture extrusion cooking has been established as the most suitable method for creating meat analogues (Schmid, 2024). This extrusion process promotes the transformation of primary ingredients, usually protein concentrates and isolates into final products with textures like the traditional meat (Schmid, 2024). It makes use of rich protein ingredients >50% (wb) and at high moisture levels >40% (wb) (Zahari et al., 2020). At the end of the extruder barrel, a long cooling die is attached, which aids in texturization of the analogues (Guyony et al., 2023; Beniwal et al., 2021).

Samard & Ryu (2019) described high moisture extrusion to be made up of four essential zones. The first zone involves hydration of the incoming protein-rich ingredients at ambient temperatures, the pressure and temperature at this zone are at minimal level, therefore the ingredients are only hydrated without any chemical or conformational effects on the materials (Wild, 2016; Chen et al., 2011). The second region is known as the kneading region, where the ingredients are blended, creating a continuous phase that takes on the consistency of a warm, dough-like material. At this region, the temperature can be mild and up to 80 °C, which causes partial denaturation of proteins (Schmid et al., 2022). The important chemical and physical changes happen in the third region called the melting zone at temperatures >120 °C and high shear conditions. During this stage, the dough is transformed into a viscous melt (Zhang et al., 2019). The proteins unfold and the secondary, tertiary and quaternary structure of the proteins are modified but the primary structure is generally kept. The last zone is the cooling section (cooling die). At this stage, the temperature is usually set to less than 80 °C. The cooling section is very

important in the formation of the structures because it controls cooling of the melt. It gradually cools the melt, and this causes the development and stabilization of the newly developed bonds. Simultaneously, the relatively lower shear condition and the absence of screw-induced mixing in the long cooling die, compared to the barrel, promote laminar flow conditions, further aiding in structure formation (See et al., 2023; Schmid et al., 2022).

2.6.1.2 Protein hydrolysates in meat analogues

Meat analogues, also called meat alternatives from plant sources with similarities to conventional meat (from animals) in terms of texture, appearance, and flavor are becoming popular in the world. This is due to their sustainability advantage over animal-based meat products. Meat alternatives have minimal energy, sodium, saturated fat, and contain no cholesterol when compared with meat burgers, thereby making them a healthier alternative for consumers (Zhou et al., 2021). While they have been shown to possess some benefits over animal meat, meat alternatives also have limitations. Zhou et al. (2021) conducted research and showed that meat alternatives from plants are digested slower than beef in the small intestine. However, it is necessary to note that the meat from animals has a balanced amino acid profile like the human body.

Plant proteins can be modified by hydrolysis to produce low molecular weight fragments with improved solubility, bio-functionality, and bioavailability (Peighambardoust et al., 2021). Hydrolyzing protein improves their nutritional composition, amino acid profile, and increases digestibility (Peighambardoust et al., 2021). Moreover, it has been established that meat analogues that contain digested proteins aid in preventing diseases, maintenance of human well-being and as such can influence human health positively. These benefits include antioxidant, immunomodulatory, antimicrobial, hypocholesterolemic, antihypertensive, and antithrombotic activities (Peighambardoust et al., 2021). Zhang et al. (2023) produced meat analogue with the

addition of hydrolyzed wheat gluten (HWG) to soy protein concentrate (SPC), which is the main ingredient. The results showed that adding HWG to SPC significantly improved the diphenyl-1-picrylhydrazyl (DPPH) and ABTS radical scavenging activities of the meat analogues. The inclusion of HWG increased protein digestibility of MAs, with higher digestibility as the ratio of HWG increased. In another study by Wang et al (2023), addition of gluten hydrolysate enhanced formation of the fibrous structure in high-moisture soy protein–gluten extrudates. The extrudates with no hydrolysate produced layered structures with some detached fibers, while those containing 1, 3 and 5% produced more distinct and thinner fibrous structures. This indicates that addition of the gluten hydrolysate enhanced visual appearance of the fibers in the extrudates. A similar study by Ji et al. (2023) indicated that addition of hydrolyzed soybean protein isolate as a plasticizer at ≤30 wt % of soybean protein improved the fibrous appearance of the meat analogue.

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CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Materials

Hemp seed protein concentrate was obtained from Manitoba Harvest Fresh Hemp Foods Ltd (Winnipeg, MB, Canada), while yellow field pea protein concentrate was obtained from Nutri-Pea Limited (Portage La Prairie, Manitoba, Canada) and all stored at –20 ºC prior to hydrolysis. Chymotrypsin, papain, and other analytical grade chemicals were purchased from Sigma-Aldrich or Thermo Fisher Corporation.

3.2 Methods

3.2.1 Enzymatic hydrolysis

The method of Asen and Aluko (2022) was used for the enzymatic hydrolysis. Each protein concentrate was mixed with double distilled water (DDQ) to obtain 5% (w/v) suspension. The beaker containing the mixture was placed on a hotplate with a magnetic stirrer equipped with an external temperature probe and pH meter for accurate temperature and pH control. The mixture was heated to the optimum temperature for each enzyme and adjusted to the optimum pH for each enzyme using 1 M HCl or 1 M NaOH under constant stirring (papain at pH 6.5 and 37 °C; chymotrypsin at pH 8.00 and 37 °C). The enzyme was introduced at this point at the ratio of papain to substrate (4:100) and chymotrypsin to substrate (1:100) based on the protein content of the concentrate. The reaction took place for 4 h under constant stirring with the pH and temperature

maintained at the optimum conditions. The reaction was terminated by adjusting to pH 4.5 using 2 M HCl followed by heating in a water bath at 95 °C for 15 min to ensure complete denaturation of the enzyme. The mixture was then cooled to room temperature and centrifuged at 5600 x g for 30 min at 4 °C. The clear supernatant was collected as the protein hydrolysate and freeze dried. The freeze-dried samples were stored at -20 °C. Extraction was done in triplicates to calculate the gross and protein yields using the equations below.

Gross yield

Gross yield is the mean of the total weight of the freeze-dried hydrolysates obtained from the three replicates of the hydrolysis. This was calculated by dividing the mean weight of the freeze-dried hydrolysates from the three replicates by the original weight of the protein concentrate and then multiplied by 100.

Protein yield

The protein yield was calculated using this formular

$$\frac{\left(weight\ of\ hydrolysate\ after\ freeze - drying \times (protein\ content\ of\ hydrolysate \div 100)\right)}{weight\ of\ concentrates \times (protein\ content\ of\ concentrate \div 100)}$$

3.2.2 Degree of hydrolysis (DH)

The DH of each protein hydrolysate was determined using the trinitrobenzene sulfonic acid method with leucine as the standard (Adler-Nissen 1979). The protein concentrates were subjected to digestion under vacuum for 24 h with 6 M HCl and the resulting digest was analyzed to determine the total amino groups, expressed as L-leucine equivalents. The DH was then calculated as the percentage ratio of the leucine equivalent of the hydrolyzed protein to the protein concentrate.

3.2.3 Protein content determination

Protein content of the hydrolysates and the meat analogues were measured using the modified Lowry method (Markwell et al., 1978). The hydrolysates, its MAs and bovine serum albumin

(BSA), which was used as the standard protein were each dissolved in 1 M NaOH to obtain 10 mg/ml and hydrated for 1 h. The protein solution was further diluted with (DDQ) to 100 µg/ml. A 1 mL aliquot of the standard and the samples were prepared in 20 - 100 µg concentration range. Then, 3 mL volume of reagent C (comprising of reagent A and reagent B in the ratio 100:1, where reagent A is made of 0.4% NaOH, 0.16% potassium sodium tartrate, 2% Na_2CO_3, and 1% Sodium dodecyl sulfate dissolved in DDQ while reagent B contains 4% $CuSO_4.5H_2O$ dissolved in DDQ) was added to 1 mL of each sample and standard and were incubated for 1 h at room temperature. After the 1 h incubation, 300 µL of reagent D (Folin Ciocalteu reagent mixed with DDQ, 1:1) was added to the samples and thoroughly vortexed. The solution was incubated for 45 min at room temperature. The absorbance of the samples was read at 660 nm with a spectrophotometer. The absorbance curve of the BSA standard was used to determine the protein concentrations in the samples.

3.2.4 Proximate composition of hydrolysates and meat analogues

The official method of the Association of Analytical Chemists was used to analyze the crude protein, dry matter, ash and moisture content of the hydrolysates and the meat analogues (Horwitz and Chemist, 2010), while the American Oil Chemists' Society standard method was used to measure the fat and crude fibre content (Mehlenbacher et al., 2020).

3.2.5 Determination of the amino acid composition of the hydrolysates and meat analogues

The hydrolysates and meat analogues were analyzed for amino acid composition using the HPLC Pico-Tag method as described by Bidlingmeyer et al. (1985) after 24 h hydrolysis of the sample with 6 M HCl solution. Performic acid oxidation was used to measure the cysteine and methionine contents (Gehrke et al., 1985), while alkaline hydrolysis was used to determine the tryptophan content (Landry & Delhaye, 1992).

3.2.6 Determination of *in vitro* enzyme inhibition activities of the hydrolysates and meat analogues

3.2.6.1 Acetylcholinesterase (AChE) inhibition

The ability of the samples to inhibit AChE activity was determined using the method of Ellman et al. (1961) as described by Asen and Aluko (2022). Each sample or the standard (galantamine) was prepared in 0.1 M phosphate buffer, pH 7.4 to a final concentration of 10 – 50 µg/mL. The substrate, 2.5 mM acetylcholine iodide (ATCI) was prepared in DDQ. AChE (5 U/ml) was prepared in the buffer, while the reaction stopper, which is 25 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) was dissolved in 50% ethanol. A 25 µL aliquot of sample/standard was mixed with 25 µL of AChE, 25 µL of ATCI and 75 µL buffer in a 96-well bottom plate. This mixture was incubated at 37 °C for 5 min. Then, DTNB (150 µL) was added to each well to terminate the reaction. The control was prepared using the same reagents, except the protein h

ydrolysate. The absorbance was read at 405 nm for 10 min with a Synergy H4 multi-mode microplate reader. The % inhibition was calculated as follows:

$$Inhibition\ (\%) = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

3.2.6.2 Angiotensin converting enzyme (ACE) inhibition

The ACE inhibition activity of the samples was measured using the method described by Udenigwe et al. (2009). Samples were dissolved in 50 mM Tris-HCl buffer containing 0.3 mM NaCl, pH 7.5 to concentration in the range of 1 – 4 mg/ml. FAPGG (0.5 mM) was the substrate, while the enzyme concentration was 0.5 U/mL. In a 96 well microplate, 50 µL of sample/standard (Captopril) was placed inside each well, 10 µL ACE was added, followed by the substrate (200 µL). Absorbance of the solution was read continuously at 345 nm for 30 min at every 60 s using a microplate reader set at 37 °C. The blank was prepared following the same method but without the

addition of the sample. The sample concentration required to decrease ACE activity by 50% was determined using non-linear regression analysis on a plot of percentage inhibition versus sample concentration with GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). ACE inhibition was calculated using the formular below:

$$1 - \left(\frac{\Delta A.min^{-1}(sample)}{\Delta A.\min^{-1}(blank)} \right) \times 100$$

3.2.6.3 Renin inhibition

Renin-inhibitory activity of the samples was assayed using the Renin Inhibitor Screening Assay Kit according to the method previously described by He et al. (2013). Samples were dissolved in 50 mM Tris-HCl, pH 8.0, containing 100 mM NaCl to 1 – 10 mg/ml final concentration. A 10 µL aliquot of sample/standard, 150 µL buffer, 10 µL of renin enzyme followed by 20 µL of renin substrate were added into the microplate wells. For the control, 160 µL of buffer, 10 µL renin and 20 µL renin substrate was used while 170 µL buffer and 20 µL renin substrate was used for the blank. The renin substrate was added in the dark to all the wells. The microplate was shaken for 10 s to mix the reactants and then incubated at 37 °C for 15 min. The assay buffer was pre-warmed to 37 °C before use. The fluorescence intensity was read using excitation wavelengths of 340 nm and emission wavelengths of 490 nm. The renin-inhibitory activity (%) was calculated using the equation below:

$$Inhibition\,(\%) = \frac{[Ac - As]}{Ac} \times 100$$

Ac = Fluorescence intensity of the control; As= Fluorescence intensity of the sample

The concentration of sample required to decrease renin activity by 50% was determined using non-linear regression analysis on a plot of percentage inhibition versus sample concentration with GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA).

3.2.6.4 α-amylase inhibition

The ability of protein hydrolysates and the meat analogue extracts to inhibit α-amylase activity was measured using the method described by Siow *et al.* (2017) with slight modifications. Sodium phosphate (0.02 M) containing 0.006 M NaCl, pH 6.9 was used as the buffer, while 1% soluble starch was used as the substrate. Samples were dissolved in the buffer to give concentrations in the range of 50 – 225 µg/mL. A 50 µL aliquot of each sample was added to a test tube alongside 50 µL of α-amylase working solution (28. 57 µg/mL). This solution was incubated at 25 °C for 10 min. After incubation, 50 µL of 1% starch (substrate, dissolved in the buffer above) was added and incubated at 25 °C for 10 mins, after which the reaction was terminated with the addition of 100 µL DNSA (dinitrosalicylic acid), a colour reagent made up of 96 mM DNSA, 2 M sodium potassium tartrate tetrahydrate and 2 M NaOH. This solution was heated in a boiling water bath at 100 °C for 5 min and allowed to cool down to room temperature, after which 1.5 mL DDQ was added to the solution. A 200 µL aliquot was taken from each test tube and transferred to the 96-well microplate and the absorbance was read at 540 nm using a microplate reader set at 25 °C. A blank reading was taken by replacing the enzyme with the buffer. This blank reading was subtracted from each well values. The equivalents of maltose released from the starch is quantified as the enzyme activity. Acarbose, a pharmacological inhibitor against α-amylase was used as the standard and analyzed following the same procedure above. The sample concentration required to reduce α-amylase activity by 50% was determined using non-linear regression analysis on a plot of percentage inhibition versus sample concentration with GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA).

The α-amylase inhibitory activity (%) was calculated using the following equation:

$$Inhibition\,(\%) = \left\{\frac{Ac - [As - Asb]}{\mathrm{Ac}}\right\} \times 100$$

Ac = Absorbance of the control; As = Absorbance of the sample; Asb = absorbance of the sample blank.

3.2.6.5 α-glucosidase inhibition

The α-glucosidase inhibitory activity of the hydrolysates and meat analogues was analyzed by the method described by Shobana *et al.* (2009) with slight modifications. Samples were dissolved in 0.1 M sodium phosphate buffer, pH 6.9 to a final concentration of 5 – 50 mg/mL. A 50 µL aliquot of each sample was placed into 96-well microplate, followed by 50 µL of freshly prepared α-glucosidase enzyme (8.33 mg/mL). The solution was incubated at 37 °C for 10 min, followed by the addition of 100 µL of the substrate, 5 mM 4-nitrophenyl a-D-glucopyranoside (PNP-glycoside) solution prepared in the buffer above. The absorbance was continuously read for 30 min at 405 nm for 30 s intervals using a microplate reader set at 37 °C. The blank was prepared using 100 µL of the buffer and 100 µL of the substrate without the addition of the enzyme. The blank value was subtracted from each well values. Acarbose was assayed following the same procedure and used as the positive control. The α-glucosidase activity was quantified by measuring the release of P-nitrophenol (absorption intensity) from the PNP-glycoside at 405 nm.

The α-glucosidase activity was calculated using the formular below

$$Inhibition\,(\%) = \left\{\frac{(Ac - Acb) - (As - Asb)}{(Ac - Acb)}\right\} \times 100$$

Ac = Absorbance of the control; As = Absorbance of the sample; Asb = absorbance of the sample blank; Acb = Absorbance of the control blank

The sample concentration required to decrease the α-glucosidase activity by 50% was determined using non-linear regression analysis on a plot of percentage inhibition versus sample concentration with GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA).

3.2.6.6 Pancreatic lipase inhibition

The inhibitory activities of the protein hydrolysates and meat analogue extracts against pancreatic lipase were analyzed as described by Tang *et al.* (2016) with slight modifications. The pancreatic lipase inhibitory activity was assayed by measuring the release of 4-methylumbellifer-one (4-MU) from the substrate, 4-methylumbelliferyl oleate (4-MUoleate). Briefly, samples were dissolved in Tris buffer (13 mM Tris-HCl, 150 mM NaCl and 1.3 mM $CaCl_2$, pH 8.0) to final concentrations of 5 – 15 mg/ml. A 25 µL aliquot of each sample/standard (orlistat) was mixed in a 96-well microplate with 225 µL substrate (0.5 mM 4-MU oleate). This solution was incubated at 37 °C for 15 min, after which 25 µL pancreatic lipase solution (3.125 U/ml) was added to initiate the enzyme reaction and was then incubated again at 37 °C for 60 min. After the incubation, the absorbance was measured at 400 nm absorption wavelength using a microplate reader which quantifies the amount of 4-methylumbelliferone released by the lipase.

The pancreatic lipase inhibitory activity (%) was calculated using the equation:

$$Inhibition\ (\%) = \left\{\frac{(Ac-As)}{(Ac\ )}\right\} \times 100$$

Ac, Absorbance of the control; As, Absorbance of the sample.

The sample concentration required to decrease the pancreatic lipase activity by 50% was determined using non-linear regression analysis on a plot of percentage inhibition versus sample concentration with GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA).

3.2.7 Antioxidant assays

3.2.7.1 DPPH radical scavenging

The DPPH scavenging activity of the hydrolysates and the meat analogues was determined following the method described by Zhang *et al.* (2009) with slight modifications. Samples were dissolved in 0.1 M sodium phosphate buffer, pH 7.0 to give assay concentration of 1 mg/mL based

on the protein content. DPPH was dissolved in 95% methanol to give assay concentration of 100 µM. A 100 µl aliquot of sample was mixed with 100 µl of the DPPH solution in a 96-well plate. This solution was incubated at room temperature for 30 min in the dark. The control for the analysis consisted of the buffer in place of the sample while glutathione (GSH) was used as the positive control and assayed following the same procedure. The DPPH radical scavenging activity (%) of the samples was determined following the equation below:

$$\%\ DPPH\ radical\ scavenging = \left(\frac{(A_{control} - A_{sample})}{A_{control}}\right) \times 100$$

Where $A_{control}$ and A_{sample} are the absorbance of the control and the sample, respectively.

3.2.7.2 Superoxide radical scavenging

The ability of the samples to scavenge superoxide radicals was determined following the method of Xie *et al.* (2008) with slight modifications. Samples or GSH were dissolved in 0.1 M NaOH to an assay concentration of 1 mg/ml. An 80 µL aliquot of sample/GSH was mixed with 80 µL of 50 mM Tris–HCl buffer, pH 8.3 (containing 1 mM EDTA) in a 96-well clear bottom plate in the dark. A 40 µL aliquot of 1.5 mM pyrogallol prepared in 10 mM HCl was added to each well to initiate the reaction. The absorbance was measured immediately at 420 nm for 4 min at room temperature. The Tris–HCl buffer was used in place of the sample as the control. The superoxide scavenging activity was calculated as follows:

$$\%\ Superoxide\ radical\ scavenging = \left(\frac{\Delta A \overset{-1}{\min}\ (Control) - \Delta A \overset{-1}{\min}(sample)}{\Delta A \overset{-1}{\min}(Control)}\right) \times 100$$

3.2.7.3 Hydroxyl radical scavenging

The hydroxyl radical scavenging was evaluated using the method described by Girgih et al. (2011). Each sample or GSH and 1,10-phenanthroline (3 mM) were dissolved separately in 0.1 M

phosphate buffer pH 7.4, while 3 mM $FeSO_4$ and hydrogen peroxide (0.01%) were separately dissolved in DDQ. A 50 µL aliquot of each sample or GSH (assay concentration of 1 mg/mL) was added to the 96-well plate, followed by the addition of 50 µL of 1,10-phenanthroline and 50 µL of $FeSO_4$. The control was prepared using 50 µL buffer, 50 µL 1,10-phenanthroline and 50 µL of FeSO4. A 50 µL aliquot of 0.01% hydrogen peroxide solution was added to each well to initiate the Fenton's reaction, covered and incubated for 1 h at 37 °C while shaking. The absorbance was read during this 1 h incubation period at 536 nm every 10 min using the microplate reader. The rate of the reaction was used to calculate the hydroxyl radical scavenging activity as follows based on changes in absorbance (ΔA):

$$HRSA(\%) = \frac{\left(A_{control} - A_{sample}\right)}{A_{control}} \times 100$$

3.2.7.4 Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) was assayed using the method described by Zhang et al. (2008) with slight modifications. To prepare the FRAP reagent, 300 mM sodium acetate buffer pH 3.6, 10 mM 4,6-tripryridyls-triazine in 40 mM HCl, and 20 mM ferric chloride were freshly mixed together in a ratio 5:1:1 (v/v/v). The FRAP reagent was preheated to 37 °C. A 40 µL aliquot of each sample or GSH (1 mg/ml assay concentration) was mixed with 200 µL of the FRAP reagent in a 96-well microplate. The absorbance was read at 593 nm relative to a reagent blank. A standard curve was prepared using ferrous sulfate (0.0625–1 mM) and the results were expressed in mM $FeSO_4$.

3.2.7.5 Metal chelation activity

The metal chelating activity of the samples was measured following a modified method of Xie et al. (2008). A 500 µL aliquot of each sample or GSH (1 mg/ml assay concentration) was premixed with 25 µL of 2 mM $FeCl_2$ and 925 µL of DDQ in a test tube. Then, 50 µL of 5 mM ferrozine

solution was added, thoroughly mixed and allowed to stand for 10 min at the room temperature. A 200 µL aliquot of each solution was taken into the 96-well plate and read at 562 nm. The control sample was prepared by replacing the sample with 500 µL DDQ. The metal chelating activity (%) was calculated using the formula below:

$$Meatl\ chelation\ activity\ (\%) = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

3.2.7.6 Inhibition of linoleic acid peroxidation

Linoleic acid oxidation inhibition was measured using the method described by Li et al. (2008). A 10 µL aliquot of each sample or GSH (1 mg/ml assay concentration) was mixed with 2.5 ml of 0.1 M sodium phosphate buffer pH 7.0 and 2.5 mL of 50 mM linoleic acid (dissolved in 99.5% ethanol). The control sample was prepared using the buffer and linoleic acid. The mixtures were kept in the dark at 60 °C for 7 days. At 24 h intervals, 100 µL of each sample was collected and mixed with 4.7 mL of 75% aqueous ethanol solution, 0.1 mL ammonium thiocyanate (30% w/v) and 0.1 mL of 0.02 M ferrous chloride (dissolved in 1 M HCL). An aliquot of 200 µL of each solution was transferred into the 96-well microplates. This was incubated for 3 min at room temperature and the color development recorded at 500 nm. An increase in the absorbance means an increase in the level of linoleic acid oxidation.

3.2.8 High-moisture extrusion cooking

Meat analogues were produced using a lab-scale co-rotating twin-screw extruder (MPF19, APV Baker Ltd., Peterborough, UK) according to the method described by Singh et al. (2024). SPC was used as the main ingredient, while pea or hemp seed protein hydrolysate was added separately in liquid form at different ratios 0, 8, 16, and 24% w/w of the SPC. Extrusion was carried out at 200 rpm screw speed and samples were collected in triplicates. The extruder barrel's temperatures at its four zones were set at 90-110-130-135 °C from the feeder to the end of the die and samples

were extruded at 65 % (w.b) moisture content (MC). A constant 0.5 kg/h (d.b.) feed rate was used for the extrusion runs, while the water flow rate was calculated based on the sample MC and the expected 65% MC in the final product. The screw profile was set using the method of Koksel and Masatcioglu (2018). A cooling die (long) with dimensions of 300 mm ×50 mm ×5 mm was fixed to the barrel at the end to ensure the development of fibrous structure in the meat analogues. This long cooling die has two zones in which the temperatures were set at 80 °C (near the barrel) and 50 °C (for the zone distant from the barrel). The die pressure and torque values were taken in triplicates during extrusion runs and used to calculate the specific mechanical energy (SME) according to Luo and Koksel (2020). The meat analogues were collected, placed in Ziplock bags, and stored at -18 °C. The samples were freeze-dried and ground into powder using a centrifugal mill (ZM200, Retsch, Haan, Germany) to give a particle size of <0.5 mm. The ground samples were extracted with water, filtered and freeze-dried for enzyme inhibition and antioxidant assays. Other portions of the samples stored in the freezer were thawed and analyzed for texture profiles and color analysis.

3.2.9 Cutting Force and Degree of Texturization of the meat analogues

The texture profile of the meat analogues was measured using a texture analyzer (TA-XT-plus, Stable Micro Systems, Godalming, UK) equipped with an A/ECB blade probe, according to the method of Guillermic et al. (2023). The cutting force in the longitudinal (parallel to the direction of the flow in the die) and transverse (direction perpendicular to the flow inside the die) were measured. Briefly, the meat analogues were first removed from the freezer and thawed to room temperature. The thawed samples were cut into 2 cm × 2 cm pieces with thickness of 5 mm, and each was placed on the texture analyzer loaded with a 30 kg cell. Each piece was cut into 75% of its actual thickness. The peak force (N) from the force versus time graph was the cutting force in

both longitudinal and transverse direction. The cutting force was performed using 2 cuts from each replicate (making six from each sample) in each direction. The degree of texturization was calculated using the ratio of the cutting force in the transverse direction to the longitudinal cutting force (Singh et al., 2024).

3.2.10 Texture Profile Analysis (TPA) of the meat analogues

The TPA of the meat analogues were determined using the method stated by Ramos-Diaz et al. (2022). The texture analyzer (TA-XT-plus, Stable Micro Systems, Godalming, UK) was equipped with a cylindrical probe (38 mm diameter) and a 30 kg load cell. A 2 cm × 2 cm cut samples of 5 mm thickness were compressed twice and the textural attributes, which include hardness, chewiness, gumminess, and springiness were determined by the force versus time graph. Hardness is defined as the peak force (N) in the first cycle of compression, while springiness, gumminess, and chewiness were calculated following the equations below (Ramos-Diaz et al., 2022).

$$Springiness = \frac{Time\ to\ reach\ the\ peak\ force\ during\ the\ \sec ond\ compression}{Time\ to\ reach\ the\ peak\ force\ during\ the\ first\ compression}$$

$$Gum\text{miness} = \frac{(Area\ of\ \sec ond\ compression)}{Area\ of\ first\ compression} \times Hardness$$

$$Chewiness = Gum \min e\,ss \times Springiness$$

3.2.11 Determination of colour profile of the meat analogues

A CM-3500d spectrophotometer (Minolta, Osaka, Japan) was used to determine the color profile of the meat analogues. The meat analogues were removed from the freezer a day prior to the analysis and allowed to thaw to room temperature. Each sample was placed on top of the color spectrophotometer light shuffle. L* (lightness), a* (greenness-redness), and b* (blueness-yellowness) were analyzed in duplicates for each replicate (six for each sample).

3.2.12 Extraction and concentration of the meat analogues

The extruded meat analogues were freeze-dried, and ground into fine powders. The proteins were extracted from the ground powders with water 3 times, and the extracts were combined and freeze-dried. The extracts were used to determine the protein content, antioxidant, bioactive properties, and bitterness intensity of the MAs.

3.2.13 Determination of bitterness intensity of the meat analogues

The bitterness intensity of the meat analogues was carried out using an electronic-tongue assay method as described by Zhang et al. (2018). Samples were dissolved in DDQ to concentrations of 0.5 - 10 mg/mL and passed through a 0.45 μm filter. The samples were brought to room temperature prior to the experiment. To calibrate and validate the e-tongue machine, bitter standard solutions were prepared with reverse osmosis water at certain concentrations, which serves as the reference points for the known bitterness levels. The standards are 0.05 mg/mL caffeine, 0.5 mg/mL acetaminophen, 0.01 mg/mL and 0.04 mg/mL quinine, 0.02 mg/mL and 0.05 mg/mL famotidine, 0.005 mg/mL loperamide hydrochloride, and 0.5 mg/mL denatonium benzoate. These standards established uniform reference points for measuring bitterness in samples through a linear regression analysis.

3.2.14 Statistical analysis

All experiments were carried out in triplicates and the data was subjected to one-way analysis of variance using SPSS software. Mean values was separated at 95% significant differences using Duncan Multiple Range Tests.

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CHAPTER FOUR

4. RESULTS AND DISCUSSIONS

4.1 Degree of Hydrolysis (DH)

The protein hydrolysates were enzymatically produced using papain and chymotrypsin to hydrolyze yellow field pea and hemp seed proteins for 4 h each. The DH results are presented in Figure 4.1 and show that papain hydrolysis had higher values for both the peas and hemp seeds while chymotrypsin hydrolysis had lower DH. The low DH values obtained for chymotrypsin hydrolysis are similar to previous reports for pea (Asen & Aluko, 2022; Humiski & Aluko, 2007), and flaxseed (Karamać et al., 2011) protein hydrolysates. This lower proteolysis rate could indicate the presence of protease inhibitors in the substrate (pea and hemp seed protein concentrates) primarily Bowman-Birk inhibitor (a double headed inhibitor), which reduced the activities of chymotrypsin (Awosika & Aluko, 2019; Mohanraj et al., 2019). This is particular for HCH, which had the lowest DH consistently for the 4 h hydrolysis. PPH had the highest DH value among all the samples and among the two papain hydrolysates with values that were consistently higher for the 4 h hydrolysis. The high DH values of papain hydrolysis could be because of its wider proteolytic specificity when compared to chymotrypsin. Papain is a cysteine endoprotease, which has a very broad specificity allowing it to cleave various peptide bonds efficiently, hence the higher

DH values, while chymotrypsin is a serine endoprotease and preferentially cleaves peptide bonds at the C-terminal side of aromatic and hydrophobic amino acids (Vogelsang-O'Dwyer et al., 2022). The broad cleavage ability of papain indicates its ability to hydrolyze substrate at different sites to produce peptides of wide range, which could have contributed to the higher DH values. The DH values obtained in this study are within the 4.01 - 20.05% range obtained for soybean protein hydrolysates (Islam et al., 2022) and lower than 5 - 30% obtained for cowpea protein hydrolysate (Mune, 2015). The higher DH values of papain hydrolysis indicates that papain is a better choice than chymotrypsin for producing protein hydrolysates from peas and hemp seeds. According to Klompong et al. (2007) and Rebeca et al. (1991), alkaline proteases showed higher activities than acid or neutral proteases, meanwhile a contrast result was obtained in this study as papain which is a neutral and slightly alkaline protease had higher DH values than the chymotrypsin which is an alkaline protease.

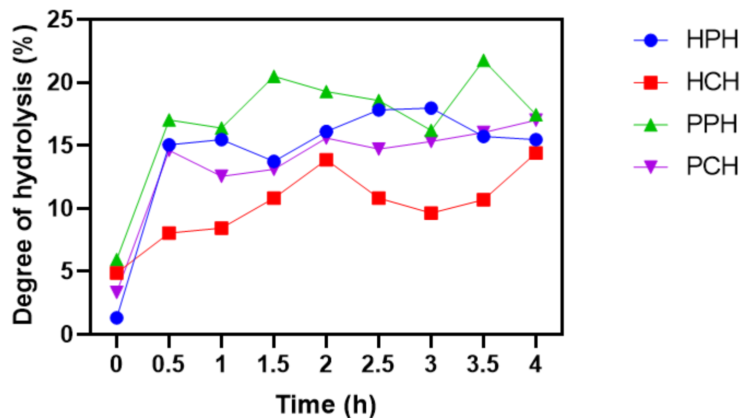


Figure 4.1: The degree of hydrolysis of the protein hydrolysates.

HPH: Hemp papain hydrolysate, HCH: Hemp chymotrypsin hydrolysate, PPH: Pea papain hydrolysate, PCH: Pea chymotrypsin hydrolysate.

4.2 Protein content and protein yield of hydrolysates

The bioactivity of proteins can be affected by their protein content and amino acid composition (Sánchez & Vázquez, 2017). The protein content and yield of the hydrolysates are shown in Table 4.1. Hydrolyzing pea proteins with chymotrypsin gave the highest protein content among all the hydrolysates, while no significant difference was observed in the hemp hydrolysates produced from either papain or chymotrypsin. Generally, the hydrolysates made from pea (PPH and PCH) had higher protein content than those from hemp, meanwhile this was not the case for protein yield. The protein yield of the hydrolysates showed significant differences based on the enzyme used. Papain hydrolysis gave higher protein yield than hydrolysis with chymotrypsin for both hemp and pea proteins, with HPH having the highest yield.

Protein yield is an indication of economic feasibility with respect to using the protein hydrolysates as commercial ingredients because higher yields will be more profitable and beneficial in new product developments (Tapal et al., 2019). During enzymatic hydrolysis, the proteins are broken down into peptides, which are then released into the soluble phase. The soluble peptides are isolated using centrifugation, which separates peptides from the insoluble constituents, especially the undigested proteins. In some instances, other constituents, which are soluble compounds (fats, sugars, free amino acids, and phenolic acids) might not be totally removed with the residue and hence can be collected with the supernatant and present in the hydrolysates (Aondona et al., 2021). The presence of these non-peptide substances can, therefore, reduce the purity level of hydrolysates. In this study, the pea hydrolysates had higher protein contents, which indicates presence of less amounts of non-peptide compounds and hence have higher purity levels than the hemp hydrolysates. Among the two pea protein hydrolysates, the chymotrypsin hydrolysate showed higher protein content than the papain hydrolysate (Table 4.1).

4.3 Proximate composition

The proximate composition of the hydrolysates was determined, and the results are presented in Table 4.1. The crude protein content ranged from 64.59% to 80.02% with HCH having the highest value. The two hydrolysates produced from chymotrypsin (HCH and PCH) had protein contents higher than those hydrolyzed with papain. This could mean that chymotrypsin had higher hydrolytic efficiency for pea and hemp seed proteins than papain. This is consistent with results from Humiski and Aluko (2007), who reported that pea protein hydrolysates produced using chymotrypsin had higher protein contents than those hydrolyzed with papain, trypsin, alcalase and flavourzyme enzymes. The protein content obtained for hemp and pea protein hydrolysates in this study is relatively similar to those obtained by Montserrat-de et al. (2023) who hydrolyzed hemp seed isolate with Alcalase and Flavourzyme (71.7% - 86.5%). While there was a significant difference $p < 0.05$ in the protein content based on the differences in protein source and enzyme used for hydrolysis, there was no significant difference in the crude fibre content of the four hydrolysates. All hydrolysates were generally low in fibre and fat. The hydrolysates had high ash content, especially PPH having ash content of 18.22%, followed by HPH (13.55%). The hydrolysates produced using chymotrypsin enzymes had lower than 10% ash content, while those from papain had higher than 13%. Ash content refers to the minerals present in the hydrolysates, the higher the ash content the higher the mineral and inorganic content. The results showed that these hydrolysates are rich in minerals, particularly those hydrolyzed with papain enzymes and therefore, could be useful for fortifying low-mineral foods to increase their mineral contents and could also improve the textural properties of the extruded MAs.

Table 4.1: Protein content, protein yield and proximate composition of hemp and pea protein hydrolysates

Proximate composition	HPH	HCH	PPH	PCH

Protein content (%)	59.41 ± 0.62^{c}	57.31 ± 0.14^{c}	67.92 ± 1.25^{b}	79.84 ± 0.14^{a}
Protein yield (%)	69.43 ± 0.02^{a}	57.65 ± 0.04^{c}	62.22 ± 0.03^{b}	54.53 ± 0.05^{d}
Moisture (%)	9.11 ± 0.1^{b}	8.395 ± 0.1^{c}	6.805 ± 0.18^{d}	11.34 ± 0.02^{a}
Dry matter (%)	90.89 ± 0.1^{c}	91.605 ± 0.1^{b}	93.195 ± 0.18^{a}	88.66 ± 0.02^{d}
Crude protein (%)	67.35 ± 0.03^{c}	80.02 ± 0.57^{a}	64.59 ± 0.86^{d}	78.03 ± 0.67^{b}
Crude fibre (%)	0.05 ± 0.03^{a}	0.04 ± 0.02^{a}	0.035 ± 0.02^{a}	0.055 ± 0.02^{a}
Fat (%)	0.24 ± 0.02^{b}	0.34 ± 0.06^{ab}	0.39 ± 0.05^{a}	0.3 ± 0.1^{ab}
Ash (%)	13.55 ± 0.03^{b}	9.43 ± 0.03^{c}	18.22 ± 0.1^{a}	7.48 ± 0.1^{d}

Mean ± standard deviation. Values with different superscripts within the same row are significantly different ($p < 0.05$). HPH: Hemp papain hydrolysate, HCH: Hemp chymotrypsin hydrolysate, PPH: Pea papain hydrolysate, PCH: Pea chymotrypsin hydrolysate.

4.4 Amino acid composition

Determining the amino acid contents of protein hydrolysates is an important measure of potential bioactivity and the nutritional composition of the hydrolysates, which is also directly related to the enzyme used (Nourmohammadi et al., 2017). The amino acid contents of the hydrolysates are presented in Table 4.2. The hydrolysates exhibited high levels of arginine ranging from 9.14 – 13.76%, asparagine, 10.15 – 11.94% and glutamine, 19.35 – 27.3%. This is in close range with the results obtained for yellow field pea protein hydrolysates (Asen & Aluko, 2022). Branched-chain amino acids (BCAA) namely valine, leucine, and isoleucine are important in prevention/management of type-2-diabetes because they are involved in regulating insulin secretion (Vanweert et al., 2022), promoting cellular senescence by activating the mammalian target of rapamycin (mTOR) pathways in cancer prevention, thereby regulating the suppression of tumor (Tamanna et al., 2014; Nakano et al., 2013), and reducing the risk of liver cirrhosis by maintaining optimal levels of serum albumin in the body (Marchesini et al., 2005; Muto et al., 2005). The hydrolysates were high in BCAA ranging between 14.41 – 16. 43%, therefore they have the potential to improve human health through normalization of insulin secretion and

prevention of excessive cell proliferation. The BCAA values obtained in this study are higher than those previously reported for pea protein hydrolysates (9.26 – 12.11%) by Asen and Aluko (2022) using pepsin, trypsin, alcalase, pancreatin, and flavourzyme enzymes. The variations could be because of the differences in the type of enzyme used for hydrolysis and its specificity.

The hydrolysates contained high levels of hydrophobic (HAA), negatively charged (NCAA) and essential (EAA) amino acids. Protein hydrolysates that are rich in HAA have high solubility in lipids and or hydrophobic substances, which enhance proton donation by facilitating interactions between peptides and free radical species (Karimi et al., 2020). Proteins are good sources of essential amino acids (EAA) that are needed in the body for necessary physiological activities (Burd et al., 2019). The antioxidant activities of the hydrolysates can be affected by the type of amino acid present in the hydrolysates (Karimi et al., 2020). Proteases are enzymes used to break proteins and peptides during hydrolysis with water. These proteases are of two types based on their positional specificity (endopeptidases and exopeptidases). Although both chymotrypsin and papain are endoproteases, their activities still differ. Papain is a cysteine endoprotease, which has a very broad specificity allowing it to cleave various peptide bonds efficiently, while chymotrypsin is a serine endoprotease and it is non-specific in action, however, preferentially cleaves peptide bonds at the C-terminal side of aromatic and hydrophobic amino acids, including tryptophan (Trp), tyrosine (Tyr), phenylalanine (Phe), and leucine (Leu) (Vogelsang-O'Dwyer et al., 2022). In this study, papain hydrolysates had higher amounts of histidine, arginine, alanine, methionine, tryptophan, sulphur containing amino acids, some essential amino acids (methionine, histidine, arginine and tryptophan) and alanine, which can be linked to papain's broad proteolytic specificity. In contrast, chymotrypsin hydrolysates had higher levels of lysine, tyrosine, leucine, and PCAA, which could be due to the narrower proteolytic specificity when compared to papain.

Table 4.2: Amino acid composition of the Hemp and Pea protein hydrolysates

Samples	HPH	HCH	PPH	PCH
HIS	3.15 ± 0.02[a]	2.32 ± 0.00[c]	3.20 ± 0.08[a]	2.69 ± 0.05[b]
SER	5.21 ± 0.00[c]	5.58 ± 0.02[a]	5.28 ± 0.01[b]	5.09 ± 0.02[d]
ARG	13.76 ± 0.04[a]	11.17 ± 0.07[b]	13.70 ± 0.24[a]	9.14 ± 0.05[c]
GLY	3.92 ± 0.01[a]	3.56 ± 0.00[d]	3.64 ± 0.04[c]	3.68 ± 0.00[b]
ASP	11.00 ± 0.05[b]	10.15 ± 0.09[c]	11.02 ± 0.23[b]	11.94 ± 0.02[a]
GLU	20.54 ± 0.09[b]	27.13 ± 0.03[a]	20.11 ± 0.27[c]	19.35 ± 0.01[d]
THR	3.45 ± 0.00[c]	3.15 ± 0.00[d]	3.53 ± 0.03[b]	3.67 ± 0.01[a]
ALA	4.03 ± 0.02[a]	2.83 ± 0.00[c]	4.01 ± 0.05[ab]	3.97 ± 0.00[b]
PRO	4.07 ± 0.00[b]	4.06 ± 0.01[b]	4.05 ± 0.01[b]	4.43 ± 0.00[a]
CYS	1.15 ± 0.02[b]	1.84 ± 0.05[a]	0.84 ± 0.01[d]	0.88 ± 0.00[c]
LYS	4.0 ± 0.01[c]	4.97 ± 0.01[b]	3.85 ± 0.06[d]	8.17 ± 0.03[a]
TYR	3.62 ± 0.04[d]	4.32 ± 0.00[a]	3.72 ± 010[c]	4.01 ± 0.03[b]
MET	2.16 ± 0.08[b]	0.60 ± 0.04[d]	2.36 ± 0.01[a]	0.92 ± 0.02[c]
VAL	4.69 ± 0.00[ab]	3.57 ± 0.01[c]	4.68 ± 0.01[b]	4.72 ± 0.00[a]
ILE	4.04 ± 0.02[b]	3.92 ± 0.03[d]	3.95 ± 0.05[c]	4.27 ± 0.02[a]
LEU	6.05 ± 0.01[d]	6.93 ± 0.02[b]	6.40 ± 0.02[c]	7.45 ± 0.03[a]
PHE	4.16 ± 0.02[c]	3.35 ± 0.04[d]	4.53 ± 0.06[b]	4.83 ± 0.00[a]
TRP	1.03 ± 0.00[b]	0.56 ± 0.00[d]	1.13 ± 0.01[a]	0.81 ± 0.00[c]
HAA	34.99 ± 0.01[c]	31.98 ± 0.04[d]	35.68 ± 0.17[b]	36.28 ± 0.02[a]
PCAA	7.14 ± 0.01[c]	7.29 ± 0.02[b]	7.05 ± 0.01[d]	10.86 ± 0.02[a]
NCAA	31.53 ± 0.13[b]	37.29 ± 0.11[a]	31.13 ± 0.49[b]	31.29 ± 0.03[b]
AAA	8.81 ± 0.02[c]	8.23 ± 0.04[d]	9.39 ± 0.15[b]	9.65 ± 0.03[a]
SCAA	3.30 ± 0.10[a]	2.45 ± 0.09[c]	3.20 ± 0.02[b]	1.80 ± 0.03[d]
BCAA	14.78 ± 0.02[c]	14.41 ± 0.07[d]	15.03 ± 0.08[b]	16.43 ± 0.01[a]
EAA	46.47 ± 0.09[b]	40.53 ± 0.12[c]	47.33 ± 0.39[a]	46.66 ± 0.08[b]

Results are presented as mean ± standard deviation

Key: HPH: Hemp papain hydrolysate, HCH: Hemp chymotrypsin hydrolysate, PP: Pea papain hydrolysate, PC: Pea chymotrypsin 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 (asparagine, aspartic acid, glutamine and glutamic acid). AAA: Aromatic amino acids (phenylalanine, tryptophan and tyrosine). SCAA: Sulphur containing amino acids (cysteine and methionine). BCAA: Branched chain amino acids (leucine, valine and isoleucine). EAA: Essential amino acids (threonine, valine, methionine, isoleucine, leucine, phenylalanine, histidine, lysine, arginine and tryptophan).

4.5 Antioxidant properties

4.5.1 DPPH radical scavenging activity

Antioxidant properties of natural substances have been evaluated by several researchers using the DPPH radical because it is stable and can be readily scavenged (Li et al., 2008). Peptides scavenge free radicals by donating hydrogen and hence terminate the radical chain reaction (Yarnpakdee et al., 2015). The DPPH radical scavenging activity (DRSA) of the MAs alongside their hydrolysates are presented in Figure 4.2a. Glutathione (GSH), which is a cellular peptide that served as the standard had the highest activity but was closely followed by SPC+HP24%. The DRSA of the MAs that contained HPH was significantly higher ($p < 0.05$) than SPC-control and every other sample. The addition of HPH, specifically 16 and 24%, led to significant ($p < 0.05$) improvement in the DRSA of the MAs when compared with SPC-control. This shows that HPH has great potential to improve the DRSA of the meat analogues particularly at higher concentration because the activity increased with increased concentration of the hydrolysate. It can also be inferred that hydrolysates have great potential in food product development. This is similar to the results of Zhang et al. (2023), who added hydrolyzed wheat gluten to MAs and reported an increase in the DRSA. However, MAs made from every other hydrolysate had lower DRSA when compared with SPC-control, which could have probably been because of an antagonistic relationship between the hydrolysates and the SPC. The MAs containing hydrolysates had higher DRSA than their hydrolysates alone, which suggests that subjecting hydrolysates to extrusion did not inactivate the activity of the peptides and may have induced structural changes to improve DRSA when compared to the hydrolysates alone. The DRSA of the MAs could have been affected by the presence of inorganic salts due to the high percentage of ash content of the hydrolysates. As reported by Al-Dabbas et al. (2007), the presence of inorganic salts can affect the DRSA of samples. K_2HPO_4 and K_2CO_3 salts increased the DRSA of Butylated hydroxytoluene. The authors said

different levels of K_2HPO_4 and K_2CO_3 salts caused changes in DPPH colour which affected the readings and resulted in inaccurate results for the actual DRSA of catechol. Since NaCl was used during the enzymatic hydrolysis of the hydrolysates to maintain the pH, this could have led to a high percentage of ash contents in the hydrolysates. This high ash percentage could have possibly negatively or positively affected the DRSA of the MAs and the hydrolysates.

Overall, MAs made with HCH, PPH, and PCH hydrolysates had lower DRSA than SPC-control, while those containing HPH had higher activity, which means that the addition of HCH, PPH, and PCH hydrolysates to soybean to produce MAs did not change or reduce the effectiveness of the soybean protein to scavenge DPPH radicals. A similar result was reported by Zhang et al. (2023), where the inclusion of wheat protein reduced the ABTS radical scavenging activity of MAs when compared to that of SPC alone. The authors attributed this to chemical cross-linking between the soybean and the wheat protein during the extrusion process and thereby resulting in MAs with a denser structure which could not scavenge free radicals. The DRSA of MAs that contained HPH increased with an increase in the level of the HPH, while a contrary result was obtained for those containing PCH. The values obtained in this study are within the range of those obtained for alcalase hemp protein hydrolysate (1.04 – 99.67), and higher than the hydrolysates produced with both alcalase and flavourzyme (1.42 – 1.83) (Montserrat-de et al., 2023).

4.5.2 Metal chelation activity (MCA)

The ability of peptides and protein hydrolysates to scavenge free radicals and chelate transition metal ions has been widely demonstrated in scientific literature (Phongthai et al., 2018). MCA is measured by determining the ability of protein hydrolysates to bind Fe^{2+} and inhibit formation of the ferrozine-Fe^{2+} complex. Fe^{2+} is a transition metal, which produces free radicals that are toxic to the human body by catalyzing lipid peroxidation rate (Bougatef et al., 2010). It is, therefore,

necessary to prevent this reaction by stabilizing the oxidized metals using chelating agents. The MCA values obtained for protein hydrolysates in this study are less than the 66 - 72% obtained by Gebrekidan (2012) at a concentration of 1 mg/ml for hemp hydrolysate produced using Proteinase K. In another study by Girgih et al. (2010), hemp seed protein hydrolysate produced with both pancreatin, and pepsin had a stronger MCA value of 72% at 1 mg/ml concentration, which is higher than the values obtained in the current study. In a similar study by Gao et al. (2021), hemp seed hydrolysate produced with protamex also had a high Fe^{2+} chelating activity of 52.9%. The differences in the MCA of these hemp seed hydrolysates could be because of the variations in the activity of the proteolytic enzymes used. However, a low Fe^{2+} chelating activity (22.87%) was also reported for the enzymatic hydrolysis of phaseolin (Carrasco-Castilla et al., 2012) while chickpea protein hydrolysate was shown to have 27.0% Cu^{2+} chelating capacity (Torres-Fuentes et al., 2011).

The potential of hydrolysates and MAs to chelate metal ions was measured and the results presented in Figure 4.2b. The results showed that GSH had the highest activity, followed closely by the MA with no protein hydrolysate (SPC-control). All the MAs made with the three levels (8 – 24%) of protein hydrolysates had lower MCA than the SPC-control, indicating that the addition of protein hydrolysates did not significantly ($p < 0.05$) improve the MCA activity of the MAs. The MCA of the MAs reduced with increased concentration of protein hydrolysates except for those made with HPH. The lower MCA of these MAs when compared with SPC-control could be because of chemical cross-linking between the soybean and the hydrolysates during the extrusion process, which could produce MAs with denser structures that could not chelate metal ions. Among the hydrolysates, PCH had the lowest MCA of 6.16%, while HPH and HCH had the highest activity of 15.60% and 13.01% respectively. MAs with SPC+HC8% and SPC+PP8% were not significantly different ($p < 0.05$) and had the highest activity (42.99% and 42.99% respectively)

among all the MAs produced with hydrolysates. These values were very similar to those of SPC-control, while SPC+PC24% had the lowest activity of 26.99%.

4.5.3 Hydroxyl radical scavenging activity

Hydroxyl radical is the most reactive of all the reactive oxygen species (ROS) because it generates hydrogen peroxide and superoxide anion when it reacts with metal ions like Fe^{2+} and Cu^{2+}, causing damage to biomolecules such as DNA and proteins (Rostami & Gharibzahedi, 2017). Therefore, removing hydroxyl radicals can help reduce the risk of damage to the cell and slow down chronic disease progression (Gharibzahedi et al., 2013; Ajibola et al., 2011). The hydroxyl radical scavenging activity of the hydrolysates and the MAs are presented in Figure 4.2c. SPC+HP16% had the highest activity, followed by a slightly lower but statistically similar activity for SPC+HC16%, which was higher than SPC-control and that of GSH. The results showed that the addition of 16% hemp protein hydrolysates from both papain and chymotrypsin produced MAs with improved activity than SPC-control. This indicates that hemp protein hydrolysates are favourable and have great potential to improve antioxidant activity of food products. In addition, the activity of SPC+PC24% was not significantly different ($p < 0.05$) from SPC-control, and both had good activities higher than GSH. In contrast, the MAs containing 8% hydrolysates had similar activities to the GSH, except for SPC+HC8% which had much lower hydroxyl radical scavenging activity. No specific trend was observed in hydroxyl radical scavenging activity for different hydrolysate concentrations. It was, however, observed that for MAs formulated with HPH and HCH, the activity peaked at 16% hydrolysate level and was higher than SPC-control, then declined at 24%. SPC+PPH had its peak activity at 8%, while SPC+PCH had the highest activity at 24%. The decline in activity of MAs at 24% hydrolysate incorporation could mean that the optimum activity had been reached at 16% hydrolysate. It could also be that the structural conformations of

complexes formed between soybean proteins and peptides at the 24% hydrolysate incorporation during extrusion did not favour optimal interactions with the hydroxyl radical. The results obtained in this study (10.46 – 52.64%) are similar to the 15 – 50% reported for mung bean protein hydrolysate by Xie et al. (2019), and higher than those reported by Olagunju et al. (2018) for pigeon pea hydrolysates (3–23%). However, higher activities have been reported for pepsin (88%) and trypsin (82%) protein hydrolysates obtained from Bambara groundnut (Arise et al., 2016).

4.5.4 Superoxide radical scavenging activity

Superoxide radicals are generated during normal metabolic activities by numerous biological reactions and are readily neutralized by endogenous antioxidants. But when produced at excessive levels that overwhelm the endogenous antioxidant system, superoxide radicals can cause lipid peroxidation, which destroys the cell membrane (Martemucci et al., 2022; Ighodaro et al., 2018). Hence, scavenging superoxide radicals is necessary to protect the cell membrane lipids, DNA and enzymes and reduce the risk of diseases related to oxidative stress (Ighodaro et al., 2018; Wang et al., 2007). The scavenging activity of the protein hydrolysates and the MAs are presented in Figure 4.2d. GSH had the highest activity followed by PPH. The activities of the MAs increased with increased level of protein hydrolysates in the MAs, and the highest activity was observed at 24% of hydrolysates. The activity of SPC-control, SPC+24%HP and SPC+24%PP were not significantly different ($p < 0.05$), and was only slightly higher than that of SPC+24%PC. This shows that the activity of the MAs improved with higher concentration of protein hydrolysates. Also, higher concentrations of hydrolysates might be required to produce MAs with improved activity than SPC-control since the highest level (24%) tested in this research led to no significant difference ($p < 0.05$) between most of the MAs and SPC-control. All the MAs had low scavenging activity at 8 and 16% hydrolysates inclusion, which was lower than the SPC-control, meaning that

the addition of hydrolysates at these levels was not optimum for producing MAs with improved scavenging activity than SPC-control. There were no significant differences ($p < 0.05$) in the activities at 16% and 24% inclusion levels for HCH and PCH in MAs. The values obtained for protein hydrolysates in this study are lower than the 35 – 69% reported for chickpea protein hydrolysates (Li et al., 2008).

4.5.5 Ferric reducing antioxidant power (FRAP)

The FRAP assay is used to determine the potential of antioxidants to directly reduce Fe^{3+} as an electron-donating activity indicator (Samaei et al., 2021). FRAP measures the potential of Fe^{3+}/ferricyanide to convert to Fe^{2+} (Gharibzahedi et al., 2013). The FRAP results for this study are presented in figure 4.2e. Four of the MAs had higher activity than SPC-control, namely, SPC+16%HP, SPC+24%HP, SPC+8%HC and SPC+8%PP, which shows that these hydrolysates have great potential for developing functional MAs with higher FRAP activity. SPC+8%HC had the highest activity, higher than GSH. The FRAP activity of the MAs containing HPH increased with an increase in the hydrolysate concentration, with 24%HPH having the highest and 8% the lowest. A similar trend was reported by Ghribi et al. (2015) and Li et al. (2008), where the reducing power of protein hydrolysate from chickpea increased with an increase in concentration, which shows a dose-dependent relationship. MAs made with PPH and PCH had their highest FRAP activity at 8% level of inclusion, while the activity declined significantly ($p < 0.05$) at 16% and 24% levels. There was no significant difference ($p < 0.05$) in the activity of the MAs that contained 16% and 24% PPH or PCH. This shows that the 8% level of pea hydrolysates inclusion was the optimum for producing MAs with strong FRAP activity. The MAs made with hemp protein hydrolysates showed good FRAP activity at all concentrations (8 – 24%) while only 8% of hydrolysates from pea protein showed good activity for both papain and chymotrypsin

hydrolysates. The addition of hemp protein hydrolysates improved the FRAP activity of MAs more than the pea hydrolysates, and only 8%PP had improved activity than SPC-control. The ability of hydrolysates to donate an electron is greatly influenced by the amount of hydrophobic amino acids (HAAs), which include methionine, leucine, histidine, tryptophan, lysine, tyrosine and isoleucine present in the peptide sequences (Ajibola et al. 2011; Pownall et al., 2010). The reducing power of hydrolysates depends on the type of hydrolysates and their concentration (Gharibzahedi et al., 2024). Therefore, the FRAP activity could be influenced by the HAA content of the samples. The higher activity of some of the MAs over SPC-control could be attributed to the high temperatures processing of the extruder, which causes unfolding and cross-linking of protein structure between SPC and the hydrolysates. As explained by Gharibzahedi et al. (2024), more peptides with electron donating ability towards Fe^{3+} were developed after pea proteins were pretreated for 30 mins at a high temperature (100 °C). Similarly, Wang et al. (2017) explained how ultrasound pretreatment of 7S and 11S soybean protein hydrolysates improved its reducing power and attributed it to unfolding of the secondary and tertiary structures and the molecular rearrangement because of the cavitation mechanism. This improvement can also be linked to the Maillard reaction that takes place during the extrusion process. Xu et al. (2018) and Zhang et al. (2014) explained how Maillard browning reaction formed melanoidins, which improved the reducing power of black bean and soybean protein hydrolysates. Stathopulos et al. (2004) reported something similar where the reducing power of soybean 7S was higher than the 11S because of the higher susceptibility of β-sheets of 7S to ultrasound waves.

4.5.6 Inhibition of linoleic acid oxidation

Lipid oxidation is a process that can produce secondary oxidation products such as aldehydes and ketones via radical chain reactions. These secondary oxidation products can cause total changes in

the quality and sensory properties (texture, color and flavor) of emulsion-based foods and edible oils (Gharibzahedi et al., 2024). Lipid oxidation causes cellular dysfunction by destroying the cell membrane, which normally protects the cell to maintain homeostasis (He et al., 2017). Bioactive peptides have the potential to inhibit lipid oxidation thereby preventing oxidative damage. One of the means of showing effectiveness as antioxidants is their ability to reduce lipid oxidation, especially in a linoleic acid model system (Sohaib et al., 2017). The ability of the hydrolysates and the MAs to inhibit linoleic acid peroxidation was determined over seven days and the results are presented in Figure 4.2f. The blank sample had the highest absorbance at 500 nm across the seven days with the exception of the first day. This overall higher absorbance indicates the rapid release of unstable linoleic acid oxidation compounds, which can degrade to form secondary metabolites as the incubation period increases. Except the blank, the absorbance of all samples decreased after the first incubation day showing inhibition of lipid oxidation. The absorbance was particularly low on days 4 and 5, which indicate the highest inhibition. The samples showed effective lipid peroxidation inhibition throughout the incubation period. The incorporation of protein hydrolysates in MAs improved their inhibitory properties as all the MAs containing protein hydrolysates had lower absorbance values than the SPC-control from day 3. In a similar report by Zhang et al. (2010), the incorporation of soy protein hydrolysates obtained from microbial proteases of Bacillus species (e.g., *B. subtilis* and *B. licheniformis*) into ground beef substantially reduced the lipid peroxidation rate.

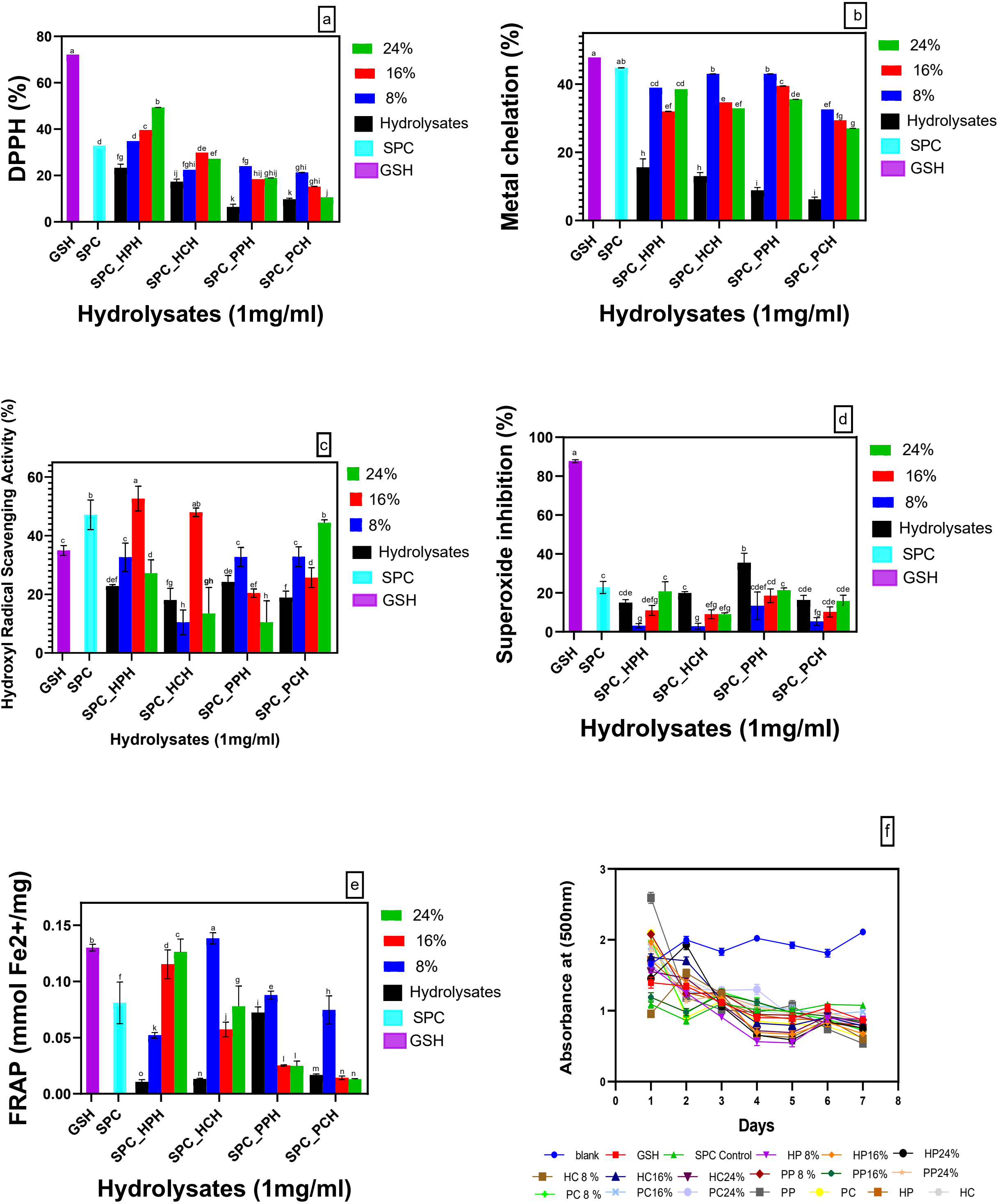


Figure 4.2 a-f: Antioxidant activities of Hemp and Pea protein hydrolysates and their MAs.

Values are mean ± standard deviation. Bars with different letters are significantly different at $p < 0.05$. Key: GSH: Glutathione, HP: Hemp papain hydrolysate, HC: Hemp chymotrypsin hydrolysate, PP: Pea papain hydrolysate, PC: Pea chymotrypsin hydrolysate, SPC Control: Soy-protein-concentrate MA, SPC+8%HP: Soy-protein-concentrate MA with 8% hemp papain hydrolysate, SPC+16%HP: Soy-protein-concentrate MA with 16% hemp papain hydrolysate, SPC+24%HP: Soy-protein-concentrate MA with 24% hemp papain hydrolysate, SPC+8%HC: Soy-protein-concentrate MA with 8% hemp chymotrypsin hydrolysate, SPC+16%HC: Soy-protein-concentrate MA with 16% hemp chymotrypsin hydrolysate, SPC+24%HC: Soy-protein-concentrate MA with 24% hemp chymotrypsin hydrolysate, SPC+8%PP: Soy-protein-concentrate MA with 8% pea papain hydrolysate, SPC+16%PP: Soy-protein-concentrate MA with 16% pea papain hydrolysate, SPC+24%PP: Soy-protein-concentrate MA with 24% pea papain hydrolysate, SPC+8%PC: Soy-protein-concentrate MA with 8% Pea chymotrypsin hydrolysate, SPC+16%PC: Soy-protein-concentrate MA with 16% pea chymotrypsin hydrolysate, SPC+24%PC: Soy-protein-concentrate MA with 24% pea chymotrypsin hydrolysate.

4.6 Enzyme inhibitory activities

4.6.1 Inhibition of α-amylase activity

α-amylase is an enzyme, which acts on starch by digesting and converting it into oligosaccharides that are further broken down by glucosidases into glucose (Visvanathan et al., 2020). The high activity of α-amylase can cause rapid digestion of starch in the intestinal tract and thereby causing glucose to be absorbed quickly into the blood stream, which could result in postprandial hyperglycemia (Bhatnagar et al., 2022). Therefore, inhibiting amylase activity can help slow glucose release, potentially preventing diabetes, a key factor in metabolic syndrome. The α-amylase inhibitory results in this study are presented in Figures 4.3. The α-amylase inhibition assay was conducted at three sample (hydrolysates or MAs + hydrolysates) concentrations (50 µg/mL, 100 µg/mL and 200 µg/mL). The results showed that all hydrolysates and MAs had α-amylase inhibitory potential. However, acarbose (a synthetic drug that was used as the standard) showed higher inhibitory activity when compared to the samples at the three concentrations. The activity of SPC-control, HPH and its MAs decreased with increased sample concentration and the optimum activity was at 50 µg/mL. The activity of PCH and its MAs increased with increase in sample concentration except for sample SPC+PC24%. However, no trend was observed for HCH, PPH and their MAs. The highest activity was observed at 50 µg/mL for most hydrolysates and

MAs. At this concentration, SPC-control had the highest inhibition among all the samples having a value of 74.94% followed closely by SPC+HP8%, SPC+HC24% and SPC+PP8%. The inclusion of protein hydrolysates did not have strong positive effects on the α-amylase inhibitory activity of the MAs as SPC-control had significantly higher ($p < 0.05$) activity in most cases. At 100 µg/mL, the activity of SPC+PC24% was higher than SPC-control while there were no significant differences ($p < 0.05$) between the activities of SPC+HP8%, SPC+PC16% and SPC-control. At 200 µg/mL, MAs made with 16% PPH, 16 and 24% PCH had improved inhibitory activity than SPC-control.

The results obtained for the hydrolysates in this study were close to the value of 75% reported for cowpea hydrolysate hydrolyzed with pepsin (Olusegun & Emmanuel, 2019). The values were also within the range reported for common bean hydrolysates (30.5 – 101.61%) produced with a combination of Flavourzyme, Alcalase, and Neutrase (Ohara et al., 2021). However, the α-amylase inhibitory values obtained in the current study are higher than those obtained for bean protein hydrolysates produced with Alcalase and Bromelain (25 – 35%) in a study by Oseguera-Toledo et al. (2015) and the ~31% at 225 µg mL^{-1} pea protein hydrolysate (Awosika & Aluko, 2019).

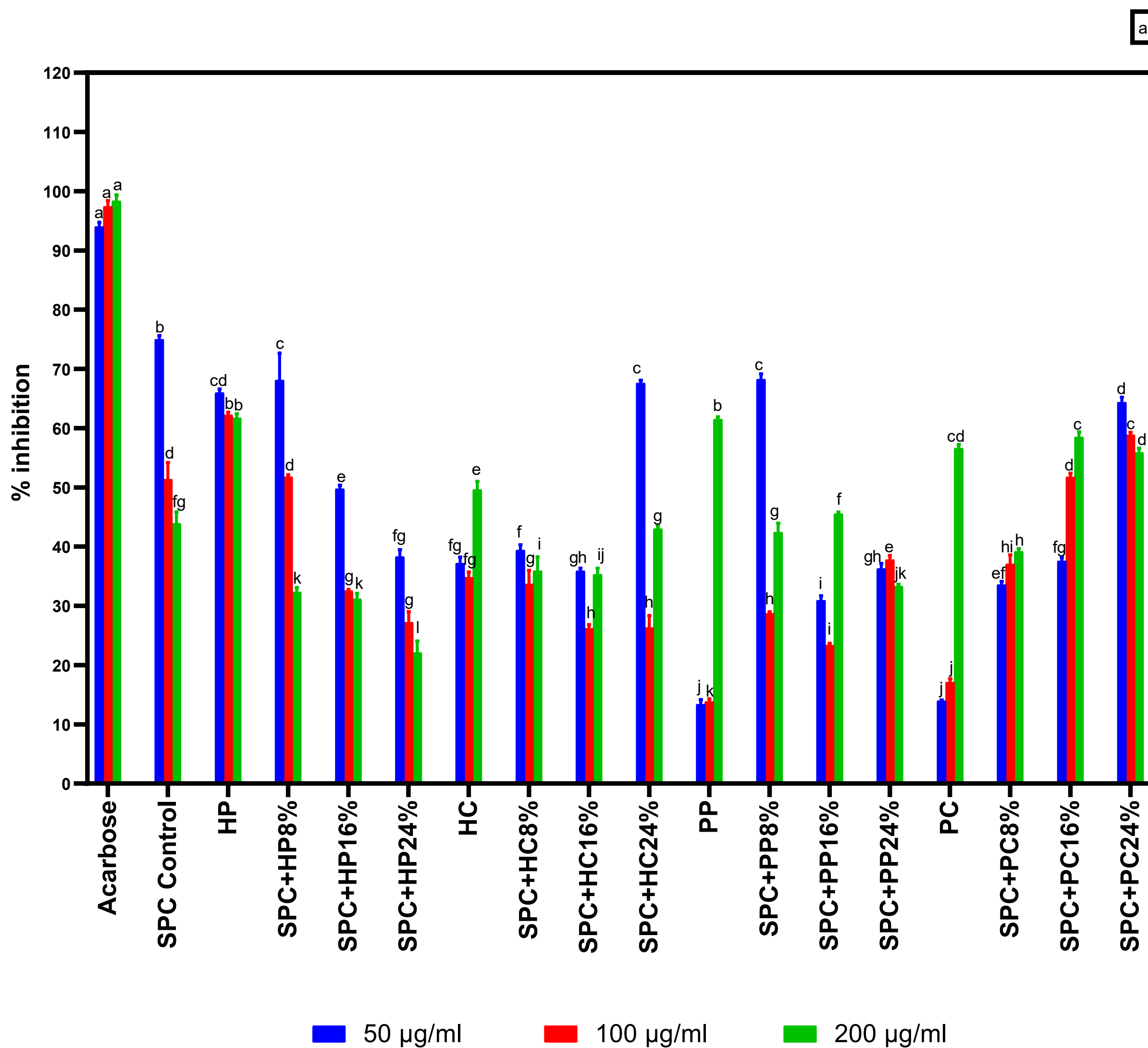


Figure 4.3: α-amylase inhibitory activity of the hydrolysates and their MAs

Values are mean ± standard deviation. Bars with different letters are significantly different at $p < 0.05$. Key: HP: Hemp papain hydrolysate, HC: Hemp chymotrypsin hydrolysate, PP: Pea papain hydrolysate, PC: Pea chymotrypsin hydrolysate, SPC Control: Soy-protein-concentrate MA, SPC+8%HP: Soy-protein-concentrate MA with 8% hemp papain hydrolysate, SPC+16%HP: Soy-protein-concentrate MA with 16% hemp papain hydrolysate, SPC+24%HP: Soy-protein-concentrate MA with 24% hemp papain hydrolysate, SPC+8%HC: Soy-protein-concentrate MA with 8% hemp chymotrypsin hydrolysate, SPC+16%HC: Soy-protein-concentrate MA with 16% hemp chymotrypsin hydrolysate, SPC+24%HC: Soy-protein-concentrate MA with 24% hemp chymotrypsin hydrolysate, SPC+8%PP: Soy-protein-concentrate MA with 8% pea papain hydrolysate, SPC+16%PP: Soy-protein-concentrate MA with 16% pea papain hydrolysate, SPC+24%PP: Soy-protein-concentrate MA with 24% pea papain hydrolysate, SPC+8%PC: Soy-

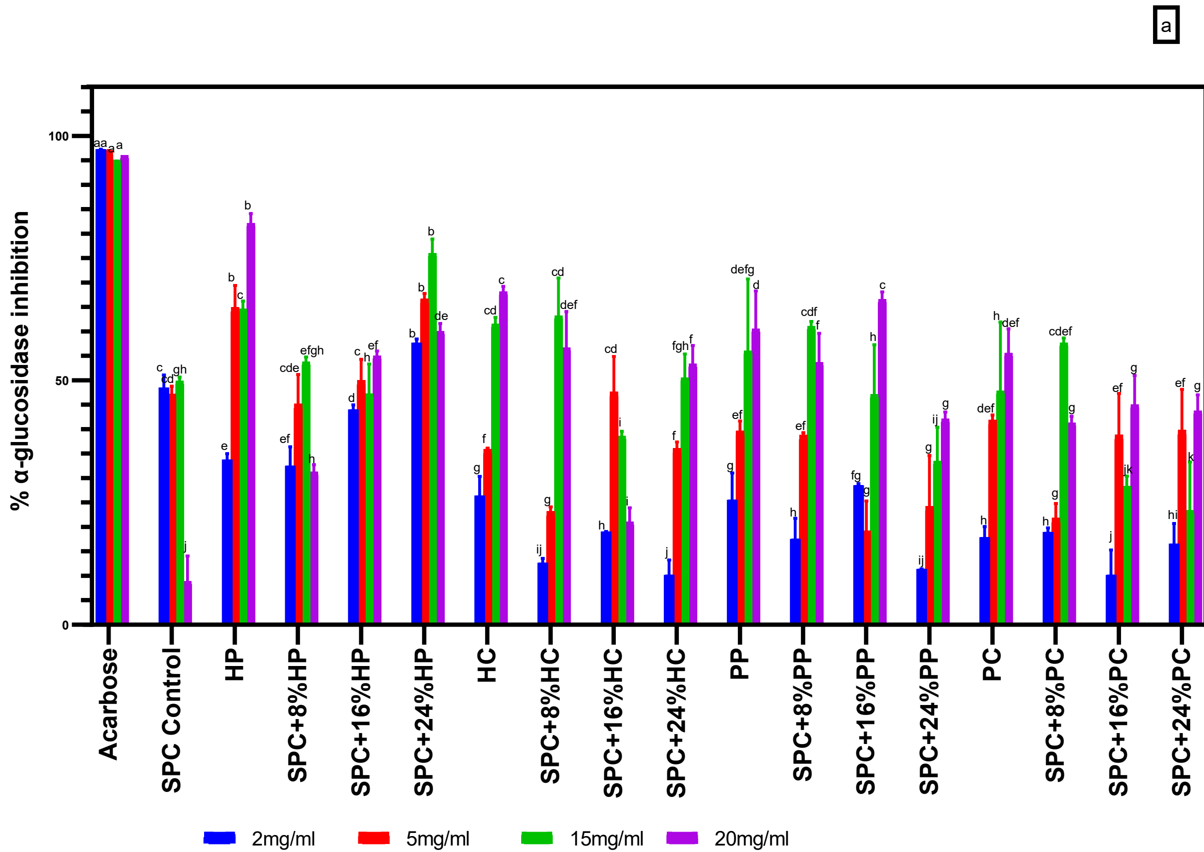
protein-concentrate MA with 8% Pea chymotrypsin hydrolysate, SPC+16%PC: Soy-protein-concentrate MA with 16% pea chymotrypsin hydrolysate, SPC+24%PC: Soy-protein-concentrate MA with 24% pea chymotrypsin hydrolysate.

4.6.2 Inhibition of α-glucosidase activity

α-glucosidase is a membrane-bound enzyme present in the small intestine epithelium. This enzyme breaks down the oligosaccharide and disaccharide products of α-amylase to release glucose, which can then be absorbed into the body (Awosika & Aluko, 2019). Inhibiting the activity of this enzyme is necessary to prevent excessive absorption of glucose into the blood stream and hence prevent/manage diabetes mellitus (Kashtoh & Baek, 2023). Inhibitory activities of the hydrolysates and MAs against α-glucosidase are shown in Figure 4.4a and b. Activities of the samples were lower than that of Acarbose, the synthetic drug used in treating diabetes. All the samples showed varied levels of inhibition against α-glucosidase. Among all the MAs studied at 20 mg/mL concentration, SPC+16%PP having 66.53% had the highest activity. At 15, 5 and 2 mg/mL, the highest activity was by SPC+24%HP (75.92%, 66.59% and 57.57% respectively) and they were all higher than SPC-control. The results show that hemp protein hydrolysates, particularly HPH, improved the activity of the MAs, with SPC+24%HP having the greatest activity. The wide variation in the inhibitory activities of samples against α-glucosidase maybe because of the differences in the peptide composition of the hydrolysates. Protein hydrolysates are composed of peptides which have different bioactivities. They exhibit various functional properties due to the sequence of their amino acids and their structures.

Considering the MAs, the optimum inhibition against α-glucosidase was obtained for SPC+24%HPH, which showed inhibition level above 50% across all concentration tested. This is probably because research has established hemp protein as a source of protein hydrolysates with enzyme inhibitory properties (Cai et al., 2023; Samaei et al., 2021; Ren et al., 2016). The results from this study are higher than those reported for common bean hydrolysates produced with

combination of proteases (Flavourzyme, Alcalase, and Neutrase) having approximately 35% inhibition (Ohara et al., 2021). The results are also similar to the 57% reported for Alcalase-digested chickpea protein hydrolysates (Quintero- Soto et al., 2021). Among all the samples, MAs made with HPH (16% and 24%) showed lower IC_{50} values than SPC-control, which indicates that these MAs are more potent than SPC-control.



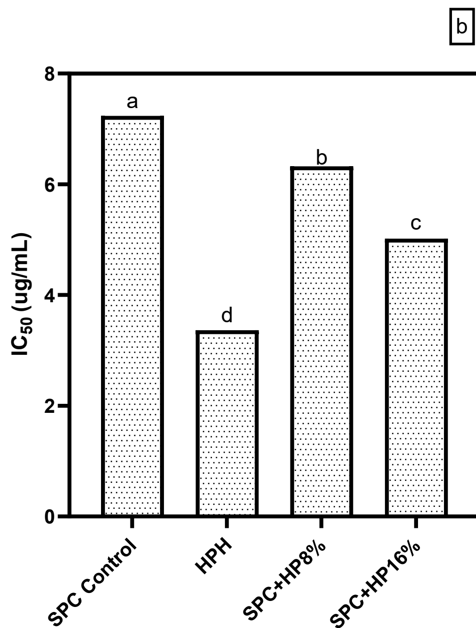


Figure 4.4a-b: α-glucosidase inhibitory activities and the IC_{50} value of the hydrolysates and their MAs.

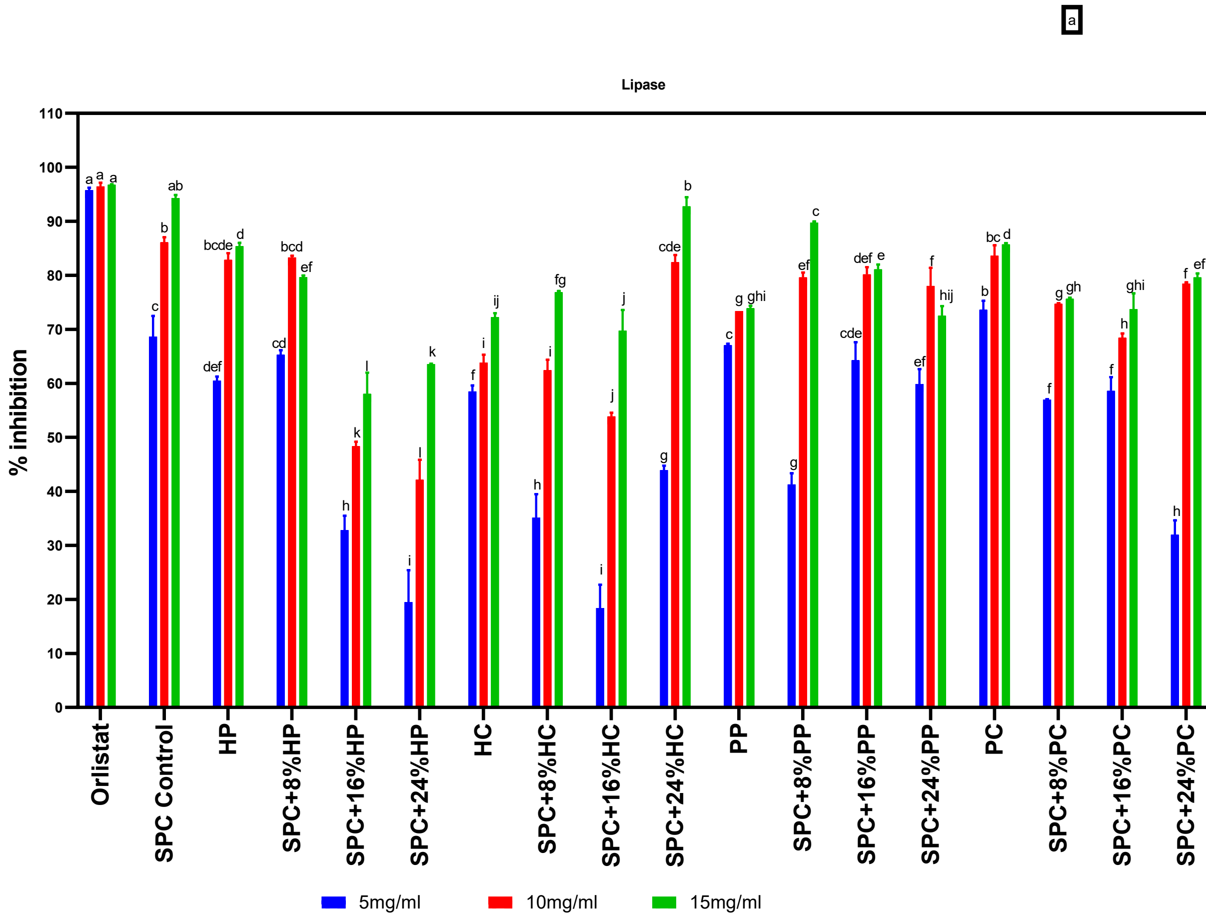
Values are mean ± standard deviation. Bars with different letters are significantly different at $p < 0.05$. Key: HP: Hemp papain hydrolysate, HC: Hemp chymotrypsin hydrolysate, PP: Pea papain hydrolysate, PC: Pea chymotrypsin hydrolysate, SPC Control: Soy-protein-concentrate MA, SPC+8%HP: Soy-protein-concentrate MA with 8% hemp papain hydrolysate, SPC+16%HP: Soy-protein-concentrate MA with 16% hemp papain hydrolysate, SPC+24%HP: Soy-protein-concentrate MA with 24% hemp papain hydrolysate, SPC+8%HC: Soy-protein-concentrate MA with 8% hemp chymotrypsin hydrolysate, SPC+16%HC: Soy-protein-concentrate MA with 16% hemp chymotrypsin hydrolysate, SPC+24%HC: Soy-protein-concentrate MA with 24% hemp chymotrypsin hydrolysate, SPC+8%PP: Soy-protein-concentrate MA with 8% pea papain hydrolysate, SPC+16%PP: Soy-protein-concentrate MA with 16% pea papain hydrolysate, SPC+24%PP: Soy-protein-concentrate MA with 24% pea papain hydrolysate, SPC+8%PC: Soy-protein-concentrate MA with 8% Pea chymotrypsin hydrolysate, SPC+16%PC: Soy-protein-concentrate MA with 16% pea chymotrypsin hydrolysate, SPC+24%PC: Soy-protein-concentrate MA with 24% pea chymotrypsin hydrolysate.

4.6.3 Pancreatic lipase inhibitory activity

Pancreatic lipase is a major enzyme which produces monoglycerides, diglycerides and free fatty acids by digesting the dietary triacylglycerols in the gastrointestinal tract (Yakaiah et al., 2021). When the activity of this enzyme is excessive, it can cause rapid absorption and digestion of lipids

leading to excess fat in the body, eventually leading to obesity, which is one of the factors contributing to the development of metabolic syndrome (Liu et al., 2020). Therefore, by inhibiting the activity of this enzyme, it has been shown to be efficient in lowering/delaying the absorption of fat and the digestion of triacylglycerol in the small intestine, hence helpful in preventing obesity and metabolic syndrome (Lunagariya et al., 2014). Ability of the MAs and the hydrolysates to inhibit pancreatic lipase is presented in Figure 4.5a & b. All samples showed strong inhibitory activity, particularly at 10 mg/mL and 15 mg/mL with more than 50% of activities. Orlistat, a synthetic drug used for therapeutic inhibition of pancreatic lipase activity, had the highest effect across the three concentrations tested. However, many other samples showed similar activity to Orlistat. Among the MAs, SPC-control had the highest activity at 10 mg/mL and 20 mg/mL (86.11% and 94.32%, respectively), indicating that the addition of protein hydrolysates in the range of 8 – 24% to develop MAs did not positively improve their activity when compared with SPC-control. SPC+8%HP had better activity than the MAs with 16% and 24% HPH. The activity decreased at 16%HP, showing that HPH activity was optimum at 8% to produce MAs with pancreatic lipase inhibition property. For HCH, all its MAs had good activity with particularly higher effect at the sample containing 24%HCH. PPH and PCH MAs also showed good activity and were higher at 8% of each. The activity of all samples increased with increasing concentrations from 5 mg/mL to 15 mg/mL showing a dose-dependent relationship, except for SPC+24%PPH, whose activity dropped at 24%. The highest activity among the MAs was observed at 15 mg/mL for SPC-control (94.32%), followed by SPC+24%HC and SPC+8%PP (92.76% and 89.77%, respectively). HPH and PCH had the highest activity among the hydrolysates. This could be because hydrolyzing hemp proteins with papain enzyme produced more peptides with inhibitory activity against pancreatic lipase than chymotrypsin, while it is contrary for pea as the

chymotrypsin hydrolysate showed higher activity. When compared with previous studies, the results obtained in this study are within the range of 23% to 87% for pinto bean peptides (Ngoh *et al*., 2017) but higher than the 20.12 – 31.08% reported for black sesame cake hydrolyzed with Flavourzyme (Chaipoot et al., 2022). The IC_{50} value was calculated and SPC had the least among all the samples, showing that it is more potent than the MAs made with protein hydrolysates. This was followed closely by HPH. Among all the samples, except SPC, only HPH and its three MAs showed IC_{50} value. The IC_{50} values obtained in this study (2.41 – 11.95 µg/mL) are higher than the 0.377 µg/mL reported for papain hempseed protein hydrolysates (Zhang et al., 2025).



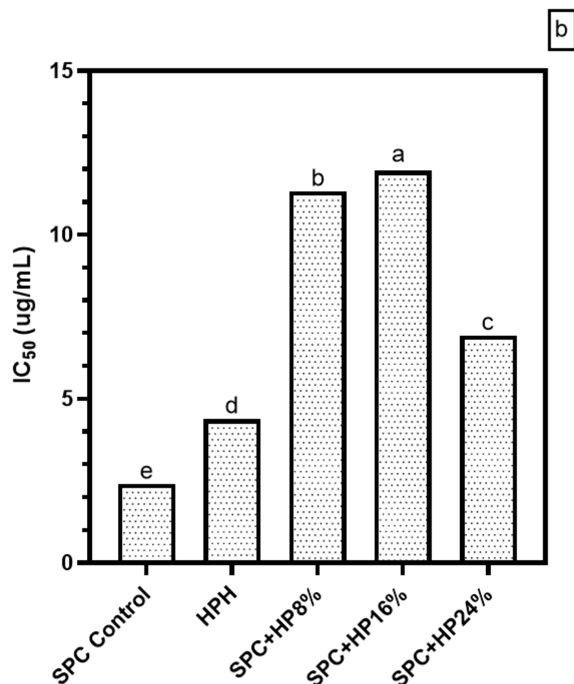


Figure 4.5a & b: Pancreatic lipase inhibitory activity and the IC_{50} values of protein hydrolysates and their MAs

Values are mean ± standard deviation. Bars with different letters are significantly different at $p < 0.05$. Key: HP: Hemp papain hydrolysate, HC: Hemp chymotrypsin hydrolysate, PP: Pea papain hydrolysate, PC: Pea chymotrypsin hydrolysate, SPC Control: Soy-protein-concentrate MA, SPC+8%HP: Soy-protein-concentrate MA with 8% hemp papain hydrolysate, SPC+16%HP: Soy-protein-concentrate MA with 16% hemp papain hydrolysate, SPC+24%HP: Soy-protein-concentrate MA with 24% hemp papain hydrolysate, SPC+8%HC: Soy-protein-concentrate MA with 8% hemp chymotrypsin hydrolysate, SPC+16%HC: Soy-protein-concentrate MA with 16% hemp chymotrypsin hydrolysate, SPC+24%HC: Soy-protein-concentrate MA with 24% hemp chymotrypsin hydrolysate, SPC+8%PP: Soy-protein-concentrate MA with 8% pea papain hydrolysate, SPC+16%PP: Soy-protein-concentrate MA with 16% pea papain hydrolysate, SPC+24%PP: Soy-protein-concentrate MA with 24% pea papain hydrolysate, SPC+8%PC: Soy-protein-concentrate MA with 8% Pea chymotrypsin hydrolysate, SPC+16%PC: Soy-protein-concentrate MA with 16% pea chymotrypsin hydrolysate, SPC+24%PC: Soy-protein-concentrate MA with 24% pea chymotrypsin hydrolysate.

4.6.4 Angiotensin-I converting enzyme (ACE) inhibitory activity

ACE inhibition serves as a key marker for identifying antihypertensive peptides in various food protein sources (Gharibzahedi et al., 2024). ACE is the enzyme responsible for converting

angiotensin-I (an inactive decapeptide) to angiotensin-II (an octapeptide), which is a very potent vasoconstrictor, and when the activity is excessive, it leads to elevated blood pressure (Ancion et al., 2019). Hypertension occurs when the systolic blood pressure is ≥140 mmHg and the diastolic blood pressure is ≥ 90 mmHg (Suo et al., 2022). The ability of the hydrolysates and their MAs to inhibit ACE activity is presented in Figure 4.6. Captopril, a synthetic drug for ACE inhibition, which was used as the standard showed activity which was only slightly higher than the activities of the MAs. However, at 1 and 4 mg/mL, there was no significant difference ($p < 0.05$) between the activity of Captopril and that of SPC-control. All the hydrolysates showed a dose-dependent relationship with their activities increasing with increased sample concentration. However, none of the MAs showed dose-dependent relationship in their activity. SPC-control had the highest activity among all the extruded MAs across all concentrations, while the other MAs also showed strong inhibitory potential above 50% inhibition across all concentrations. The inclusion of protein hydrolysates resulted in MAs with slightly lower inhibitory activities when compared with SPC-control. This could have been caused by antagonistic relationship between soybean proteins and the protein hydrolysates. The results of this study are similar to the 74 - 99.5% ACE inhibition as reported by de Oliveira Filho et al. (2021) for cottonseed byproduct protein hydrolysate produced with alcalase. The authors also stated that heat treatment of the samples improved the ACE inhibitory activity to 99.5% when compared to non-heat treatment. The value obtained in this study is also similar to the 70.51% reported for pigeon pea protein hydrolyzed with a mixture of pepsin, trypsin and chymotrypsin (Ratnayani et al., 2019).

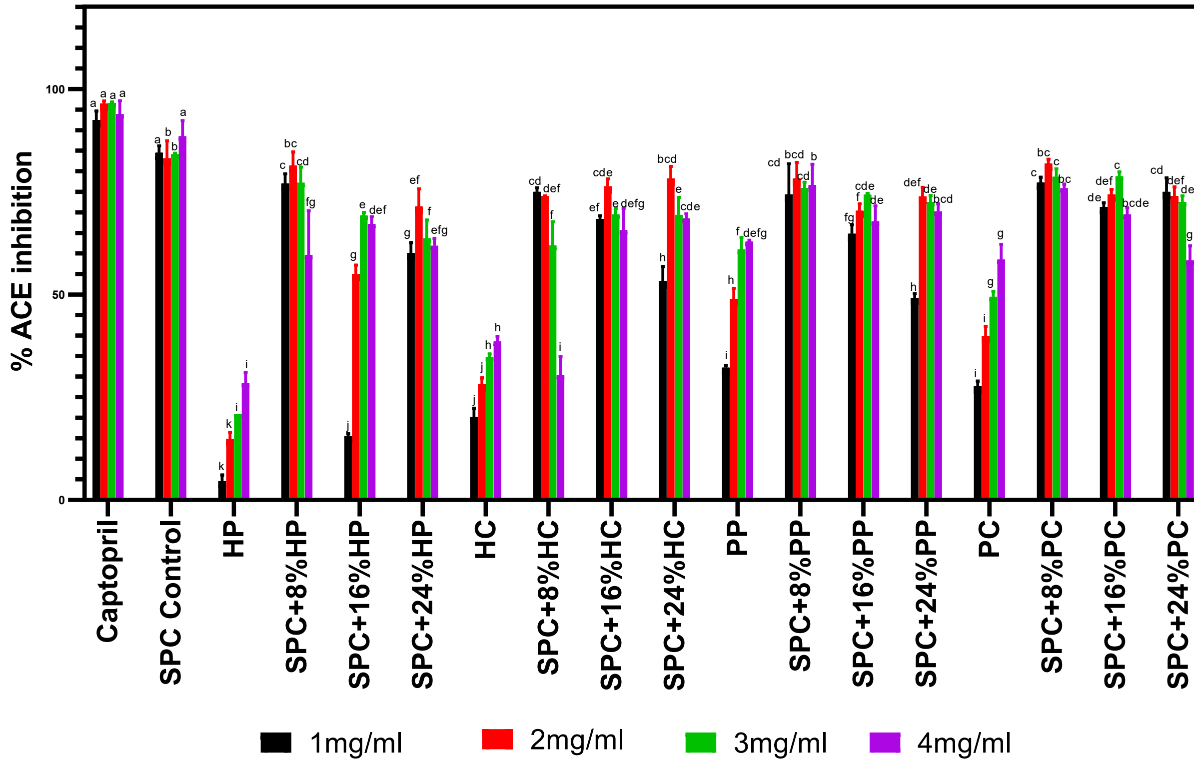


Figure 4.6: ACE inhibitory activity of the protein hydrolysates and their MAs

Values are mean ± standard deviation. Bars with different letters are significantly different at $p < 0.05$. Key: HP: Hemp papain hydrolysate, HC: Hemp chymotrypsin hydrolysate, PP: Pea papain hydrolysate, PC: Pea chymotrypsin hydrolysate, SPC Control: Soy-protein-concentrate MA, SPC+8%HP: Soy-protein-concentrate MA with 8% hemp papain hydrolysate, SPC+16%HP: Soy-protein-concentrate MA with 16% hemp papain hydrolysate, SPC+24%HP: Soy-protein-concentrate MA with 24% hemp papain hydrolysate, SPC+8%HC: Soy-protein-concentrate MA with 8% hemp chymotrypsin hydrolysate, SPC+16%HC: Soy-protein-concentrate MA with 16% hemp chymotrypsin hydrolysate, SPC+24%HC: Soy-protein-concentrate MA with 24% hemp chymotrypsin hydrolysate, SPC+8%PP: Soy-protein-concentrate MA with 8% pea papain hydrolysate, SPC+16%PP: Soy-protein-concentrate MA with 16% pea papain hydrolysate, SPC+24%PP: Soy-protein-concentrate MA with 24% pea papain hydrolysate, SPC+8%PC: Soy-protein-concentrate MA with 8% Pea chymotrypsin hydrolysate, SPC+16%PC: Soy-protein-concentrate MA with 16% pea chymotrypsin hydrolysate, SPC+24%PC: Soy-protein-concentrate MA with 24% pea chymotrypsin hydrolysate.

4.6.5 Renin inhibitory activity

Renin is another enzyme, which plays a crucial role in regulating blood pressure. It is the first enzyme involved in the renin-angiotensin system and is responsible for converting

angiotensinogen into angiotensin-I (Aluko, 2019). Hence, by inhibiting the activity of this enzyme, blood pressure can also be regulated. The renin inhibitory activities of the samples are presented in Figure 4.7. Only a few samples showed inhibitory activities such as HCH, PPH, PCH and the MAs that contained 16 – 24% HPH and HCH. SPC+8%HC, SPC+8%PP and SPC+24%PP showed low activities. Among all the samples, PPH had the highest activity at 4 mg/mL. The samples with renin-inhibitory activity showed dose-dependent activity, because their activity increased with increased sample concentration. SPC-control showed no inhibition against this enzyme, however, when protein hydrolysates, specifically those made from hempseed protein (16 – 24%) was added, it significantly ($p < 0.05$) improved the inhibitory activities of the MAs. This shows that hemp protein hydrolysates have great potential in developing MAs with renin inhibitory activity. Only MAs made with hemp protein hydrolysates from both papain and chymotrypsin inhibited renin activity while the pea protein hydrolysates did not have significant ($p < 0.05$) activities when incorporated to develop MAs. This shows that hemp protein could be a potential source of renin inhibitory peptides in food product development. On the contrary, PPH had the highest activity even more than the hempseed hydrolysate and its MAs. However, the extruded products containing PPH showed no renin inhibition, probably indicating inactivation during the extrusion process. The results from this study are similar to those obtained for other plant protein hydrolysates such as 43.72% for *Moringa oleifera* (Ma et al., 2021), 5.82 – 31.37% for Lima bean (Ciau-Solís et al., 2018) and 40.5 - 70.9% for oat protein hydrolysates (Bleakley et al., 2017).

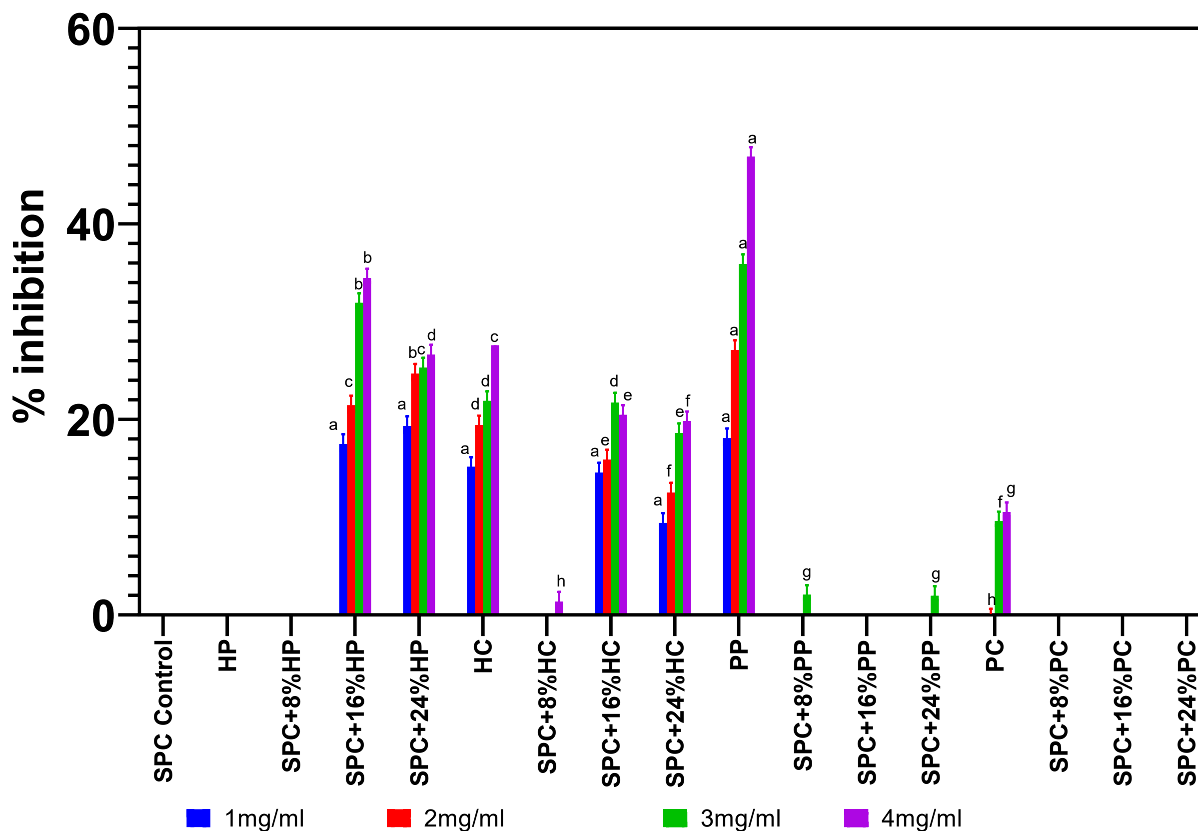


Figure 4.7: Renin inhibitory activity of the hydrolysates and its MAs

Values are mean ± standard deviation. Bars with different letters are significantly different at $p < 0.05$. Key: HP: Hemp papain hydrolysate, HC: Hemp chymotrypsin hydrolysate, PP: Pea papain hydrolysate, PC: Pea chymotrypsin hydrolysate, SPC Control: Soy-protein-concentrate MA, SPC+8%HP: Soy-protein-concentrate MA with 8% hemp papain hydrolysate, SPC+16%HP: Soy-protein-concentrate MA with 16% hemp papain hydrolysate, SPC+24%HP: Soy-protein-concentrate MA with 24% hemp papain hydrolysate, SPC+8%HC: Soy-protein-concentrate MA with 8% hemp chymotrypsin hydrolysate, SPC+16%HC: Soy-protein-concentrate MA with 16% hemp chymotrypsin hydrolysate, SPC+24%HC: Soy-protein-concentrate MA with 24% hemp chymotrypsin hydrolysate, SPC+8%PP: Soy-protein-concentrate MA with 8% pea papain hydrolysate, SPC+16%PP: Soy-protein-concentrate MA with 16% pea papain hydrolysate, SPC+24%PP: Soy-protein-concentrate MA with 24% pea papain hydrolysate, SPC+8%PC: Soy-protein-concentrate MA with 8% Pea chymotrypsin hydrolysate, SPC+16%PC: Soy-protein-concentrate MA with 16% pea chymotrypsin hydrolysate, SPC+24%PC: Soy-protein-concentrate MA with 24% pea chymotrypsin hydrolysate.

4.6.6 Acetylcholinesterase (AChE) inhibitory activities

AChE is an enzyme, which catalyzes the breakdown of acetylcholine (ACh) into acetic acid and choline. ACh is involved in signals transmission in the brain and supports nerve functioning. ACh is a neurotransmitter, which supports memory function and regulates muscle control in the central nervous system of mammals (Asen & Aluko, 2022). Excessive activity of AChE in breaking down ACh causes early termination of cholinergic transmission, hence a reduction in nerve transmission and memory capacity (Asen & Aluko, 2022). Figures 4.8 show the AChE inhibitory activity of the samples. All the hydrolysates and MAs inhibited AChE activity at the three concentrations tested, however, with more potency at 10 µg/mL. Almost all samples showed higher inhibitory activity than Galantamine, a potent AChE inhibitory drug. MAs made with pea protein hydrolysates (PPH and PCH) showed higher activity than those made with hemp protein hydrolysates. Most of the MAs made with pea protein hydrolysates had higher inhibitory activity than SPC-control particularly at 10 µg/mL and 20 µg/mL. This could be interpreted to mean that pea protein hydrolysates effectively improved the activity of the MAs or there was synergistic relationship between soybean protein and pea protein hydrolysates which enhanced their inhibitory activities. The results obtained in this study are higher than the ≤20% reported for pea protein hydrolysate (Asen & Aluko, 2022).

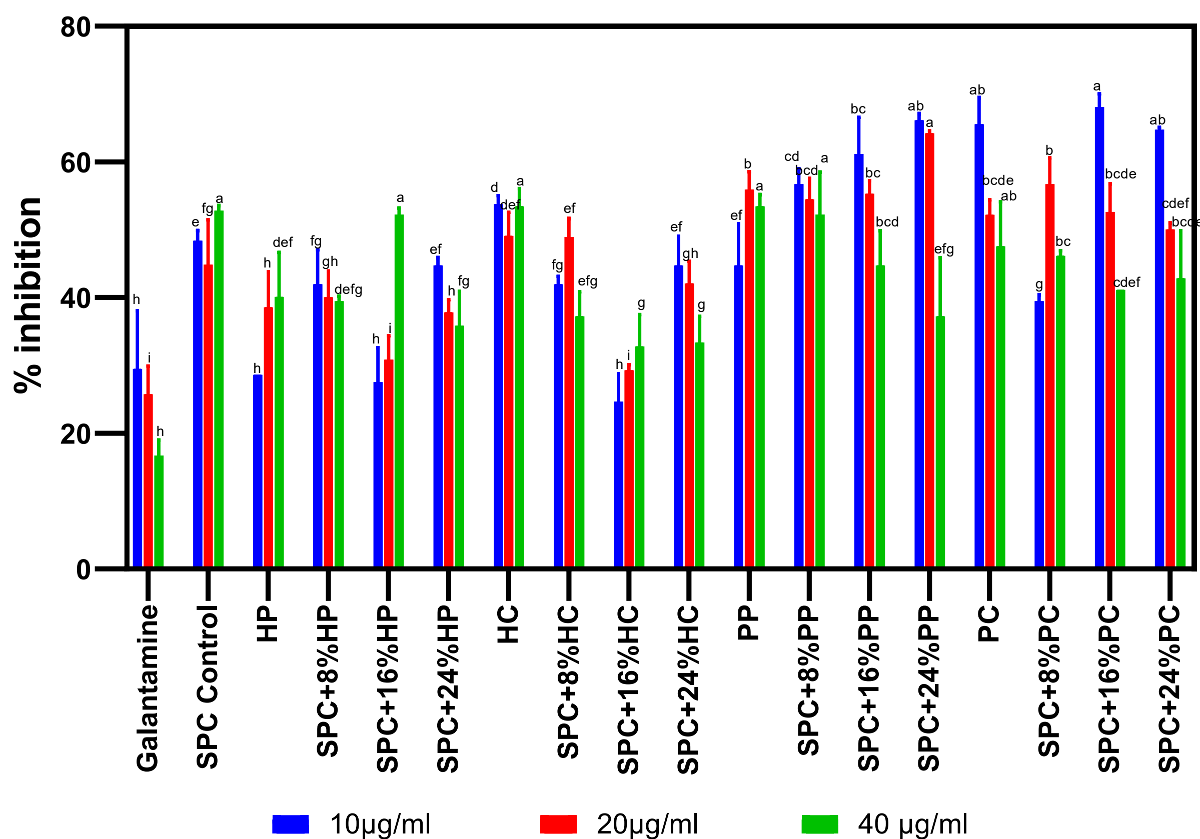


Figure 4.8: AChE inhibitory activities of the hydrolysates and their MAs

Values are mean ± standard deviation. Bars with different letters are significantly different at $p < 0.05$. Key: HP: Hemp papain hydrolysate, HC: Hemp chymotrypsin hydrolysate, PP: Pea papain hydrolysate, PC: Pea chymotrypsin hydrolysate, SPC Control: Soy-protein-concentrate MA, SPC+8%HP: Soy-protein-concentrate MA with 8% hemp papain hydrolysate, SPC+16%HP: Soy-protein-concentrate MA with 16% hemp papain hydrolysate, SPC+24%HP: Soy-protein-concentrate MA with 24% hemp papain hydrolysate, SPC+8%HC: Soy-protein-concentrate MA with 8% hemp chymotrypsin hydrolysate, SPC+16%HC: Soy-protein-concentrate MA with 16% hemp chymotrypsin hydrolysate, SPC+24%HC: Soy-protein-concentrate MA with 24% hemp chymotrypsin hydrolysate, SPC+8%PP: Soy-protein-concentrate MA with 8% pea papain hydrolysate, SPC+16%PP: Soy-protein-concentrate MA with 16% pea papain hydrolysate, SPC+24%PP: Soy-protein-concentrate MA with 24% pea papain hydrolysate, SPC+8%PC: Soy-protein-concentrate MA with 8% Pea chymotrypsin hydrolysate, SPC+16%PC: Soy-protein-concentrate MA with 16% pea chymotrypsin hydrolysate, SPC+24%PC: Soy-protein-concentrate MA with 24% pea chymotrypsin hydrolysate.

4.7 Extrusion system parameters

The extrusion system parameters (die pressure, torque and specific mechanical energy (SME)) are presented in Table 4.3. SME refers to the quantity of energy that is generated by friction between the raw ingredient mixture and the screws, which is exerted from the extruder motor into the melt that is being extruded (Kantrong et al., 2018). The SME values during extrusion decreased with an increase in the percentage of the hydrolysate content for all the MAs. The decrease in SME with increasing hydrolysate content indicates a reduction in energy requirements to process the raw materials during extrusion. This can be linked to decrease in the melt viscosity and therefore, lesser mechanical energy will be required to transport the melt through the die (Fang et al., 2014). The results show that addition of protein hydrolysates resulted in a decrease in the mixture viscosity on a concentration dependent basis. The SME values obtained in this study (78.78 - 113.00 Wh/kg) were the same order of magnitude as those obtained by Ghanghas et al. (2024) for soy-based MAs (47.4 - 64.6 Wh/kg). This decrease in SME values with increasing hydrolysates was also observed with the torque and die pressure values. The values decreased with increase in the hydrolysate content. The decrease in melt viscosity could be because of the low starch in the mixture, since the addition of hydrolysate would increase the protein content of the mixture, hence lower starch content and hence the lower viscosity of the melt (Webb et al., 2020). The protein content of the SPC was 78.53% (db) while the MAs ranges from 76.7 – 93.1% (db). MAs made with HPH had the lowest SME, torque and die pressure values, while those made with PP hydrolysates were generally higher. This lower SME by the inclusion of hydrolysate could offer the benefits of producing MAs with acceptable fibrous structure while reducing the SME of the extrusion process. Reduction in SME of the extruder will positively influence the screws, liners and the motor, cause less wear and tear thereby prolonging the life span of the important parts, hence making it economical (Hou et al., 2020; Mateen & Singh, 2023).

Table 4.3: Torque, die pressure and specific mechanical energy (SME) of the extrusion process

Sample name	**Torque (%**	**Die pressure (Kpa)**	**SME (Wh/kg)**
SPC control	11.67 ± 0.58 [g]	880 ± 74.83 [f]	83.56 ± 3.38 [g]
SPC_8%HPH	12.33 ± 0.5 [f]	600 ± 66.67 [g]	88.33 ± 3.38 [f]
SPC_16%HPH	11.89 ± 0.60 [fg]	555 ± 68.49 [gh]	85.15 ± 4.06 [fg]
SPC_24%HPH	11 ± 0.00 [h]	500 ± 66.67 [h]	78.78 ± 0.00 [h]
SPC_8%HCH	14.11 ± 0.33 [c]	1233.33 ± 66.67 [c]	101.07 ± 2.25 [c]
SPC_16%HCH	13 ± 0.00 [e]	1055.56 ± 49.69 [d]	93.11 ± 0.00 [e]
SPC_24%HCH	12 ± 0.00 [fg]	966.67 ± 47.14 [e]	85.95 ± 0.00 [fg]
SPC_8%PPH	15.67 ± 0.5 [a]	1377.78 ± 91.62 [a]	112.21 ± 3.38 [a]
SPC_16%PPH	15.78 ± 0.44 [a]	1311.11 ± 99.38 [ab]	113.00 ± 2.98 [a]
SPC_24%PPH	13.56 ± 1.01 [d]	1077.78 ± 41.57 [d]	97.09 ± 6.85 [d]
SPC_8%PCH	14.9 ± 0.32 [b]	1255.56 ± 68.49 [bc]	107.43 ± 0.00 [b]
SPC_16%PCH	13.67 ± 1.00 [cd]	1133.33 ± 92.70 [d]	97.88 ± 6.75 [cd]
SPC_24%PCH	13.33 ± 0.5 [de]	1077.78 ± 78.57 [d]	95.49 ± 3.38 [de]

Values are mean ± standard deviation. Columns with different letters are significantly different at $p < 0.05$. Key: SPC Control: Soy-protein-concentrate MA, SPC+8%HP: Soy-protein-concentrate MA with 8% hemp papain hydrolysate, SPC+16%HP: Soy-protein-concentrate MA with 16% hemp papain hydrolysate, SPC+24%HP: Soy-protein-concentrate MA with 24% hemp papain hydrolysate, SPC+8%HC: Soy-protein-concentrate MA with 8% hemp chymotrypsin hydrolysate, SPC+16%HC: Soy-protein-concentrate MA with 16% hemp chymotrypsin hydrolysate, SPC+24%HC: Soy-protein-concentrate MA with 24% hemp chymotrypsin hydrolysate, SPC+8%PP: Soy-protein-concentrate MA with 8% pea papain hydrolysate, SPC+16%PP: Soy-protein-concentrate MA with 16% pea papain hydrolysate, SPC+24%PP: Soy-protein-concentrate MA with 24% pea papain hydrolysate, SPC+8%PC: Soy-protein-concentrate MA with 8% Pea chymotrypsin hydrolysate, SPC+16%PC: Soy-protein-concentrate MA with 16% pea chymotrypsin hydrolysate, SPC+24%PC: Soy-protein-concentrate MA with 24% pea chymotrypsin hydrolysate.

4.8 Cutting force and degree of texturization

The cutting force in both longitudinal (F_L) and transverse (F_T) directions is presented in Figure 4.9a and b. These values are used to determine the degree of texturization and the fiber arrangement for high moisture MAs (Singh et al., 2024). The F_L value in this study ranged from 5.66 to 10.94 N and the F_T ranged from 6.27 to 9.12 N. These values are similar to (3.73 - 6.00 N) and (3.97 - 5.79 N) obtained for soy-based MAs for F_L and F_T respectively (Ghanghas et al., 2024). The F_L and F_T for SPC+HPH increased with increasing hydrolysates concentration while the opposite result was obtained for SPC+HCH. The increase observed for SPC+HPH could be because of the

higher protein concentration at higher hydrolysate concentration in the melt leading to a stronger network strength and more protein-protein interactions. However, the reduction observed with SPC+HCH at high concentrations could mean that 8%HCH is the optimum concentration needed to produce MAs with high cutting strength. This means the addition of HCH is optimal up to a particular limit beyond which it becomes detrimental to protein network formation. However, since concentrations lower than 8% of HCH were not tested, it is difficult to validate this interpretation. Zhang et al. (2023) reported that excessive addition of wheat gluten hydrolysate decreased the fibrousness of MAs, which is similar to the outcome obtained for the incorporation of >8% HCH in the current work.

SPC+PPH and SPC+PCH showed a trend in their F_T, which was different from the above trend. The highest cutting force was obtained at 24% hydrolysate, followed by 8%, while the MAs with 16% hydrolysate had the lowest F_T. The F_L showed a different trend with increases at high hydrolysate contents, however, no significant difference ($p < 0.05$) was observed for SPC+PCH at 16 and 24%, while for SPC+PPH, the F_L was highest at 16% and reduced at 24%. These variability in the cutting strength shows that using different types and/or concentrations of protein hydrolysates in the production of MAs can result in MAs with different range of cutting strength depending on the target consumer. For example, elderly people would prefer meat with lower cutting strength, which will be easier to chew while younger people might prefer a higher cutting strength (Liu et al., 2022). Therefore, results from this work show that the cutting strength of MAs can be tailored to suit different consumers by selecting the appropriate hydrolysate concentration, which is consistent with the suggestion that the type and concentration of hydrolysates determine the cutting force of MAs.

The degree of texturization result of the MAs is presented in Figure 4.9c. Values >1 indicate a good fibrous structure (Zhang et al., 2023), meaning that it will be more difficult to cut the meat in a longitudinal direction compared to transverse direction. This is because the fibrous structures are more aligned towards the longitudinal direction of the extrudate flow. The values obtained in this study ranged from 0.67 – 1.20. Four of the MAs had their values higher than 1, which show the presence of fiber structure in the flow direction of the die. The degree of texturization of the MAs containing HPH decreased with increasing hydrolysate concentration, while no significant difference ($p < 0.05$) was observed for those made with HCH. Those made with pea hydrolysate had the highest value at 8% (1.18 and 1.20) for SPC+PPH and SPC+PCH respectively. These values are very close to the degree of texturization for chicken breast (1.26) and drumstick (1.23), and this infers that they are very similar in structure to the chicken meat. Compared to previous reports, the values obtained in this report are similar to the 0.90 – 1.2 reported by Lee et al., (2023), and 1.06 – 1.13 reported for MAs made with sunflower meal by Singh et al. (2024). All the MAs had their highest degree of texturization at 8% hydrolysate, which could mean that at this concentration, the fibrous structure are more aligned in the direction of the flow of the die. The values for SPC and SPC+8%PPH were not significantly different ($p < 0.05$) from each other and were higher than 1, however SPC+8%PCH had the highest value and was the only sample higher than SPC, therefore the addition of PCH at 8% improved the fibrous structure more than the SPC. SPC+HCH generally had the lowest values across the three concentrations, which shows HCH fibrous orientation consisted mainly of perpendicular fibrous structure.

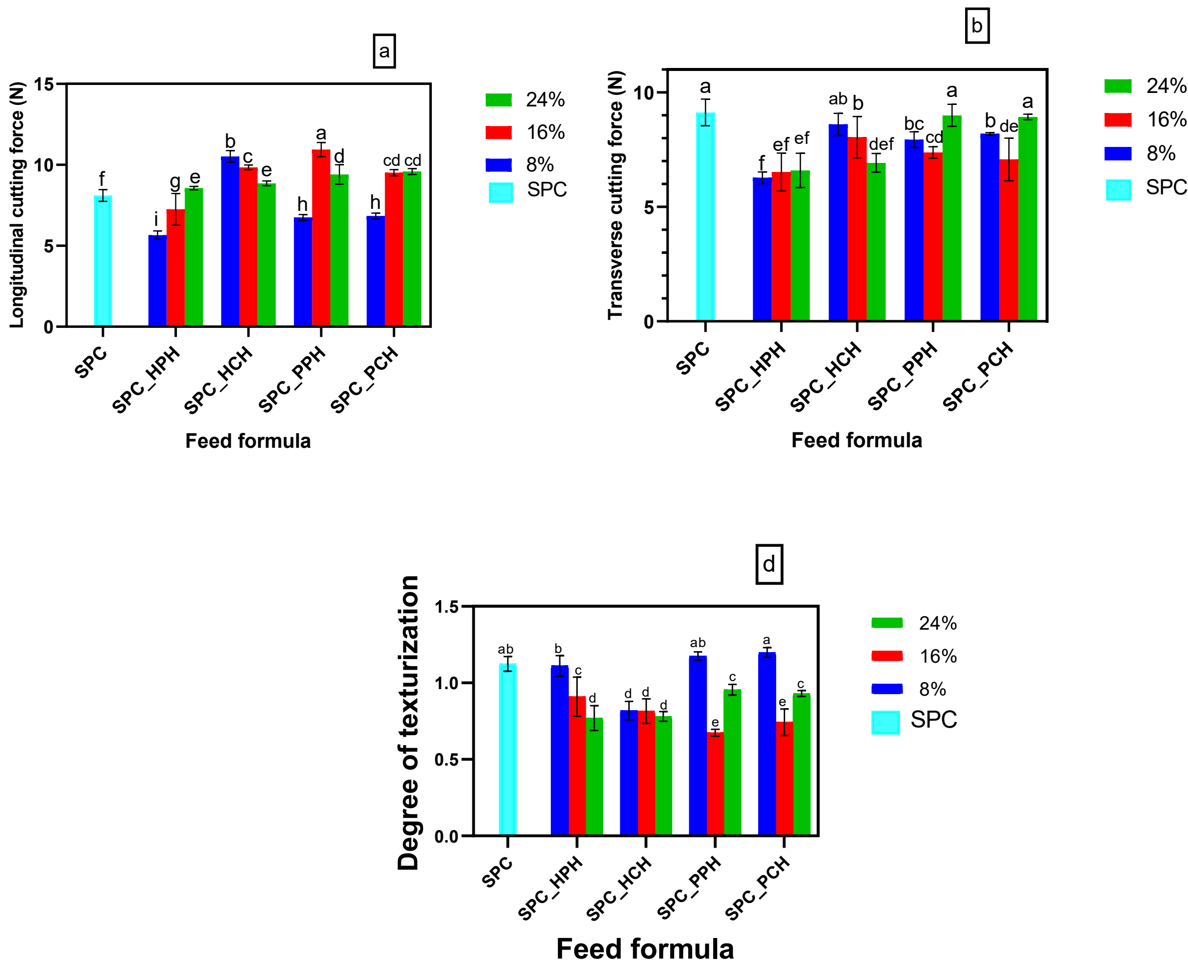


Figure 4.9a – c: Longitudinal, transverse cutting force and the degree of texturization of the MAs

Values are mean ± standard deviation. Bars with different letters are significantly different at $p < 0.05$. Key: SPC Control: Soy-protein-concentrate MA, SPC+HPH: Soy-protein-concentrate MA with hemp papain hydrolysate, SPC+HCH: Soy-protein-concentrate MA with hemp chymotrypsin hydrolysate, SPC+PPH: Soy-protein-concentrate MA with pea papain hydrolysate, SPC+PCH: Soy-protein-concentrate MA with pea chymotrypsin hydrolysate.

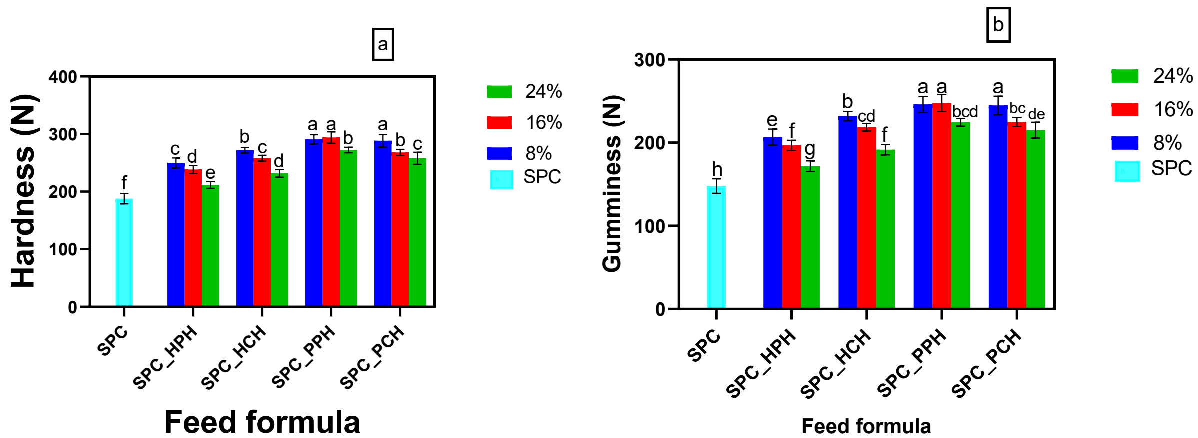
4.9 Textural profile of the MAs

Textural profiles of the MAs are presented in Figure 4.10a - d. Generally, the addition of protein hydrolysates increased the hardness values of the MAs when compared to SPC, which had the least hardness value. This shows that protein hydrolysates can be used to improve the hardness and other textural properties of MAs. The higher hardness value in the MAs containing hydrolysates could be influenced by the high levels of minerals (ash) present in the hydrolysates

as discussed under the proximate composition. salt authors have established how high levels of salt can increase the absorption of water in the extrusion process which eventually improved the fibrous structure. Dinani et al. (2023) researched the effects of two different types of salts (NaCl and $CaCl_2$) on the tensile strength, macrostructures and microstructures of soybean protein-based meat simulants. The researchers said NaCl possibly increased the cross-link bonds and fiber structures. In another research, Peng et al. (2021) found that the hardness and chewiness of low moisture extruded wheat gluten increased with an increase in NaCl. Xia et al. (2024) observed that NaCl with 1% – 2% content favoured the textural qualities of high-moisture textured yeast protein at the extrusion temperature of 180 ◦C.

The hardness values for the MAs in this study reduced as the protein hydrolysate concentration increased. This is consistent with the torque, SME and die pressure values, which decreased with increasing protein hydrolysate concentration. The hardness values in this study ranged between 187.84 to 294.11 N, which is higher than those reported by Ghanghas et al. (2024) for MAs made from soybean protein and lower than 137.8-531.8 N reported by Ramos-Diaz et al. (2023) for MAs made from native lupin flour and lupin protein concentrate/isolate. Since chewiness and gumminess values are directly related to hardness, decrease in chewiness and gumminess values with respect to the hydrolysate concentration can be expected. The decrease in these values with increasing hydrolysate concentration could be because of the lower torque and SME, which could have been caused by lower melt viscosity. The lower melt viscosity not only reduces torque and SME input by enabling a smoother melt flow through the barrel (Díaz et al., 2022) but also promotes a more extended flow profile in the long cooling die, aligning the fibers in a longitudinal direction (Diaz et al., 2022; Wittek et al., 2021; Osen et al., 2014). A 16% or higher hydrolysate concentration could be beneficial in producing MAs for elderly people since they will prefer softer

meat that is easier to chew. However, when planning to make MAs with harder structure, then 8% hydrolysates could be considered. The reduction in hardness, chewiness and gumminess with increasing hydrolysate concentration could also be because hydrolysates are composed of free amino acids and small peptides, which can interact with soy protein molecules to form a more flexible gel network structure, therefore producing softer meat textures (Zhang et al., 2023). The fibrous arrangement of MAs can be disrupted at high concentrations of protein hydrolysates to produce more brittle and compact structure with less mechanical anisotropy (Ji et al., 2023). The authors also reported that hydrolysates increased water binding ability and thereby increased the hydration capacity to produce MAs with less dense structure and therefore decreased gumminess. Thus, the reduced hardness makes it easy to bite and chew the MAs (Ji et al., 2023).



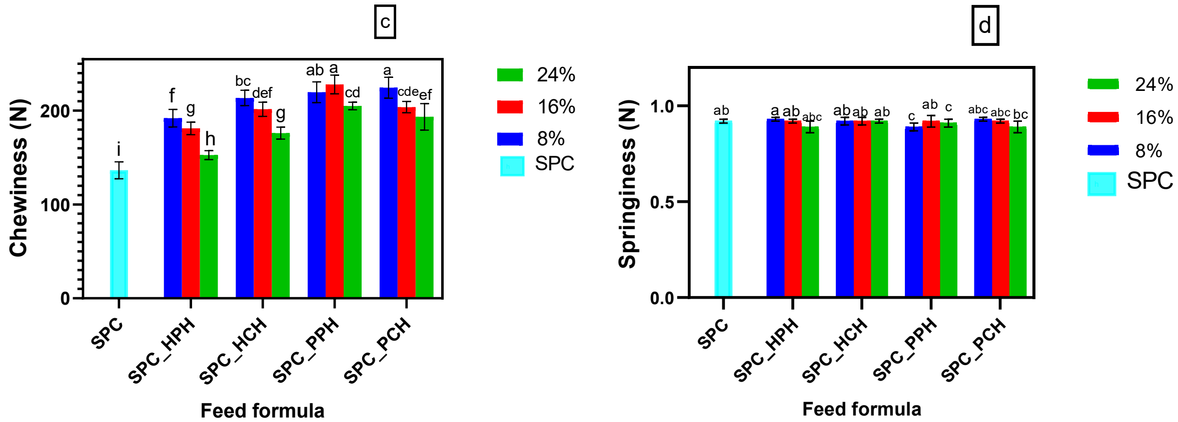


Figure 4.10a – d: Hardness, gumminess, chewiness and the springiness values of the MAs

Values are mean ± standard deviation. Bars with different letters are significantly different at $p < 0.05$. Key: SPC Control: Soy-protein-concentrate MA, SPC+HPH: Soy-protein-concentrate MA with hemp papain hydrolysate, SPC+HCH: Soy-protein-concentrate MA with hemp chymotrypsin hydrolysate, SPC+PPH: Soy-protein-concentrate MA with pea papain hydrolysate, SPC+PCH: Soy-protein-concentrate MA with pea chymotrypsin hydrolysate.

4.10 Color analysis

The L* value indicates the brightness of the color (0 - dark, while 100 – white). As presented in Table 4.4, L* values of the MAs decreased with increasing hydrolysates concentration, indicating darker colors, which means the extruded products become darker at higher level of hydrolysates. Meanwhile, the MAs that contained 8%PPH had higher L* value than SPC-control, hence it is brighter than the control sample. Generally, the inclusion of hempseed protein hydrolysate darkens the MAs than the inclusion of yellow field pea hydrolysates. This is because field peas are naturally brighter than the polyphenol-enriched hemp seed. Generally, the inclusion of 8% hydrolysate did not result in significant differences ($p < 0.05$) in the L* values between the MAs and the SPC-control except for SPC+8%HPH. The darker color of the extruded products could be because of the different reactions happening during the extrusion process, such as Maillard reaction, hydrolysis, caramelization, and nonenzymatic reaction such as pigment oxidation (Camire et al., 1990). Meanwhile for a* (green to red, negative values indicate green while positive values

indicate red) and b* (blue to yellow, negative values indicate blue while positive values indicate yellow). All the samples had positive a* and b* values, which indicate redness and yellowness. Both values increased with increasing hydrolysates concentration.

Table 4.4: L*, a* and b* values of the extruded MAs

Sample	L*	a*	b*
SPC	48.91 ± 0.45 [b]	6.22 ± 0.17 [g]	18.72 ± 0.10 [g]
SPC_HPH_8%	45.62 ± 0.46 [hi]	5.85 ± 0.39 [h]	17.78 ± 0.56 [h]
SPC_HPH_16%	44.68 ± 0.25 [j]	6.22 ± 026 [g]	18.09 ± 0.29 [h]
SPC_HPH_24%	43.45 ± 0.25 [k]	6.43 ± 0.11 [g]	18.04 ± 0.19 [h]
SPC_HCH_8%	48.17 ± 0.49 [c]	7.33 ± 0.18 [e]	19.81 ± 0.21 [ef]
SPC_HCH_16%	45.76 ± 0.35 [h]	7.69 ± 0.06	20.00 ± 0.14 [ae]
SPC_HCH_24%	45.32 ± 0.18 [i]	7.99 ± 0.10 [d]	20.58 ± 0.25 [d]
SPC_PPH_8%	49.40 ± 0.16[a]	7.65 ± 0.09 [d]	20.47 ± 0.10 [d]
SPC_PPH_16%	48.09 ± 0.30 [cd]	8.49 ± 0.12 [c]	21.52 ± 0.24 [c]
SPC_PPH_24%	47.15 ± 0.20 [fg]	9.31 ± 0.15 [a]	22.48 ± 0.35 [a]
SPC_PCH_8%	47.70 ± 0.51 [de]	7.10 ± 0.18 [f]	19.63 ± 0.34 [f]
SPC_PCH_16%	47.49 ± 0.36 [ef]	8.43 ± 0.13 [c]	21.35 ± 0.19 [c]
SPC_PCH_24%	46.77 ± 0.36 [g]	9.09 ± 0.26 [b]	21.97 ± 0.46 [b]

Values are mean ± standard deviation. Columns with different letters are significantly different at $p < 0.05$. Key: SPC Control: Soy-protein-concentrate MA, SPC+8%HP: Soy-protein-concentrate MA with 8% hemp papain hydrolysate, SPC+16%HP: Soy-protein-concentrate MA with 16% hemp papain hydrolysate, SPC+24%HP: Soy-protein-concentrate MA with 24% hemp papain hydrolysate, SPC+8%HC: Soy-protein-concentrate MA with 8% hemp chymotrypsin hydrolysate, SPC+16%HC: Soy-protein-concentrate MA with 16% hemp chymotrypsin hydrolysate, SPC+24%HC: Soy-protein-concentrate MA with 24% hemp chymotrypsin hydrolysate, SPC+8%PP: Soy-protein-concentrate MA with 8% pea papain hydrolysate, SPC+16%PP: Soy-protein-concentrate MA with 16% pea papain hydrolysate, SPC+24%PP: Soy-protein-concentrate MA with 24% pea papain hydrolysate, SPC+8%PC: Soy-protein-concentrate MA with 8% Pea chymotrypsin hydrolysate, SPC+16%PC: Soy-protein-concentrate MA with 16% pea chymotrypsin hydrolysate, SPC+24%PC: Soy-protein-concentrate MA with 24% pea chymotrypsin hydrolysate.

4.11 Bitterness intensity

One of the factors that limit the application of protein hydrolysates in food products development is their bitter taste. Arteaga et al. (2020) explained that the astringency and bitterness of protein hydrolysates were part of the important reasons that limit the acceptance of sensory group members. The bitterness-intensity of the MAs and the hydrolysates are presented below in Figure

4.11. All the MAs containing protein hydrolysates had less bitter taste when compared to SPC-control. Among the hydrolysates, hemp seed protein hydrolysates had higher bitterness, which may be due to the presence of higher levels of polyphenolic compounds when compared to the pea protein hydrolysates. Among the hemp seed protein hydrolysates, the chymotrypsin hydrolyzed had the highest bitterness, indicating that hydrolyzing hemp seed protein with chymotrypsin resulted in more bitter peptides than the papain hydrolyzed, while no significant difference ($p < 0.05$) was observed in pea hydrolysates for both papain and chymotrypsin hydrolyzed. The use of protein hydrolysates with SPC to produce MAs significantly ($p < 0.05$) reduced the bitterness of the MAs in comparison to SPC-control and their hydrolysates alone, which may be due to dilution effect when the hydrolysate is mixed with SPC. From this result, it shows that SPC-control had a higher bitter taste than MAs containing hydrolysates even at 24% hydrolysate concentrations. The bitter taste of each MA increased with increasing hydrolysate concentrations, which is expected. The results show that the use of protein hydrolysates in food processing such as extrusion cooking has tendency to lower the bitterness intensity of the hydrolysates, probably due to changes in peptide structure during the processing. This shows the potential for application of protein hydrolysates in extruded food production with less bitter taste. The bitterness scores obtained in this research (8.18 – 15.67) are higher than the 0.2 – 5.5 obtained by Xia et al. (2022) for pea protein hydrolysates from different debittering methods and the 0.5 – 5.1 reported for two-step enzyme-assisted soybean protein hydrolysates (Tong et al., 2020). However, the values from the current work are similar to the <15 reported for glycated beef protein hydrolysates (Zhang et al., 2019).

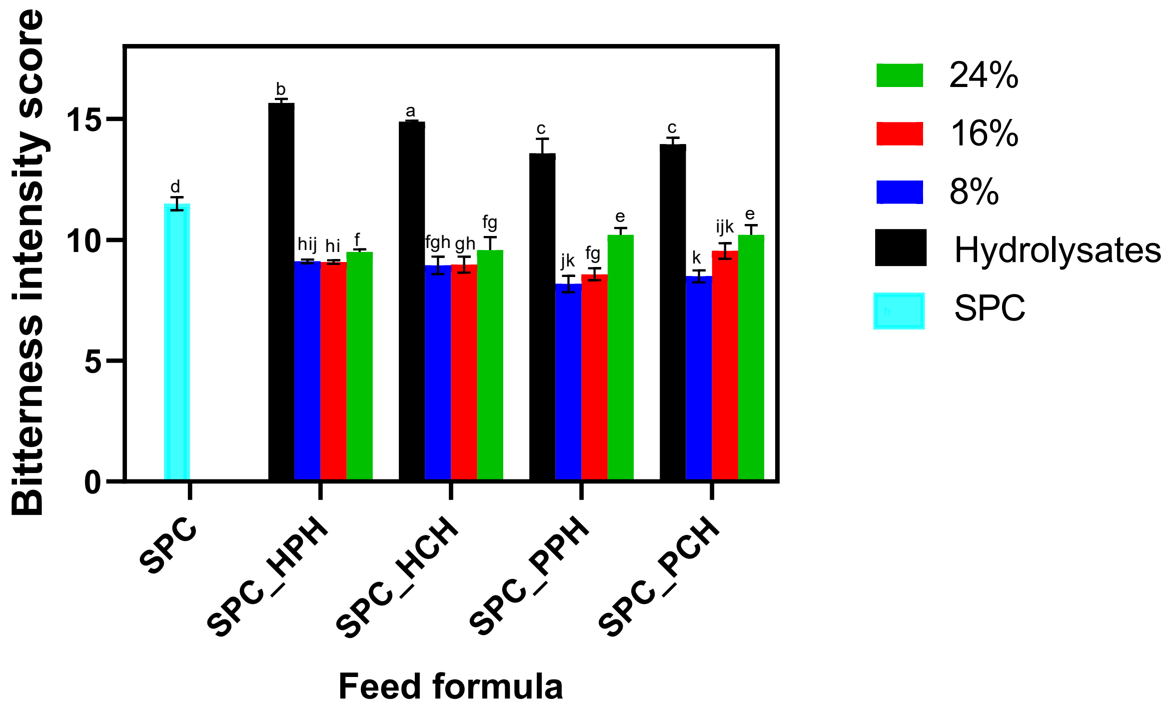


Figure 4.11: Bitterness intensity of protein hydrolysates and MAs

Values are mean ± standard deviation. Bars with different letters are significantly different at $p < 0.05$. Key: HPH: Hemp papain hydrolysate, HCH: Hemp chymotrypsin hydrolysate, PPH: Pea papain hydrolysate, PCH: Pea chymotrypsin hydrolysate, SPC Control: Soy-protein-concentrate MA, SPC+8%HP: Soy-protein-concentrate MA with 8% hemp papain hydrolysate, SPC+16%HP: Soy-protein-concentrate MA with 16% hemp papain hydrolysate, SPC+24%HP: Soy-protein-concentrate MA with 24% hemp papain hydrolysate, SPC+8%HC: Soy-protein-concentrate MA with 8% hemp chymotrypsin hydrolysate, SPC+16%HC: Soy-protein-concentrate MA with 16% hemp chymotrypsin hydrolysate, SPC+24%HC: Soy-protein-concentrate MA with 24% hemp chymotrypsin hydrolysate, SPC+8%PP: Soy-protein-concentrate MA with 8% pea papain hydrolysate, SPC+16%PP: Soy-protein-concentrate MA with 16% pea papain hydrolysate, SPC+24%PP: Soy-protein-concentrate MA with 24% pea papain hydrolysate, SPC+8%PC: Soy-protein-concentrate MA with 8% Pea chymotrypsin hydrolysate, SPC+16%PC: Soy-protein-concentrate MA with 16% pea chymotrypsin hydrolysate, SPC+24%PC: Soy-protein-concentrate MA with 24% pea chymotrypsin hydrolysate.

CHAPTER FIVE

5. CONCLUSION

This study has shown the possibility of utilizing plant protein hydrolysates in food product development, particularly through extrusion. The study revealed that both the hemp seed and yellow field peas are good sources of active protein hydrolysates when hydrolyzed with papain and chymotrypsin. The protein hydrolysates favorably improved the antioxidant properties, enzyme inhibitory activity and the textural quality of some of the MAs that were formulated with the protein hydrolysates. Of particular interest is the hemp seed protein hydrolysates, which showed better bioactive properties when incorporated in MAs. MAs that contained hempseed protein hydrolysates had better antioxidant activity than those formulated with pea protein hydrolysates. The bitter taste of protein hydrolysates is one of the long-standing limitations to their application in food product development. Interestingly, SPC-control had a higher bitterness score than all the MAs formulated with hydrolysates, showing that the use of protein hydrolysate could improve acceptability. SPC+24%HP had the highest α-glucosidase activity, and its consumption may be useful in reducing blood sugar levels. The MAs that contained pea protein hydrolysates had higher Acetylcholinesterase inhibitory activity than those with hempseed protein hydrolysates, while only MAs made with hempseed protein hydrolysates showed renin inhibition. The extruded MAs had low L* values and became darker at higher level of hydrolysates. Generally, SPC+HPH had the lowest cutting forces in both longitudinal and transverse direction which could make it more suitable as a preference for aged people, while SPC+HCH, SPC+PPH and SPC+PCH all showed higher cutting forces values in both directions. All MAs had the highest degree of texturization at 8% of hydrolysate concentration, which drastically decreased at 16% hydrolysates, except SPC+HCH whose values were generally low across the three concentrations, indicating that 8% hydrolysate is an optimum concentration required to produce MAs with more fiber

structure aligned towards the flow of the die. The texture profile (hardness, gumminess, chewiness and springiness) decreased with increase in the hydrolysate concentration except for SPC+PPH whose values were highest at 16% hydrolysate. All MAs with hemp seed hydrolysates generally had lesser TPA values than those with pea hydrolysates indicating that hemp seed hydrolysates are suitable for making softer MAs while peas might be suitable for making MAs with harder texture. In summary, protein hydrolysates derived from hemp seed and yellow field pea seeds showed great potential for developing MAs with improved texture, sensory and health benefits, including antioxidant, antihypertensive, and antidiabetic potentials, making them ideal ingredients for food product development.

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