Functional and Bioactive Properties of Protein Isolates and Hydrolysates from Camelina and Sophia Seed Meals

By

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ABSTRACT

Camelina and sophia meals, often seen as by-products from these oilseed crops, are promising resources in the battle against food waste due to their high nutrient content. These by-products, rich in proteins, fibers, essential amino acids, and antioxidants, could be repurposed as functional food ingredients or nutraceuticals, thus enhancing food's nutritional value. These meals align with sustainability goals, as they can decrease waste and promote a circular economy. The rising consumer interest in plant-based diets and alternative protein sources further highlights their potential as valuable food products. Further research could position these oilseed by-products as crucial players in reducing food waste and creating nutritious, functional, and sustainable food products.

This research involved the acquisition of camelina protein isolates (CPI) and sophia protein isolates (SPI) through the application of traditional and ultrasonic-assisted extraction methods. We conducted a detailed examination of the protein isolates using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and explored their functional characteristics. It was notable that the application of ultrasonic extraction resulted in a substantial improvement in the functional properties of both camelina and sophia protein isolates, which included increased protein solubility, water holding capacity, oil absorption capacity, as well as emulsifying and foaming properties.

Furthermore, this study focused on using sophia and camelina seed meals to produce protein hydrolysates through enzymatic processes (Alcalase and Flavourzyme). The antioxidant activities of these protein hydrolysates were examined using *in vitro* methods

such as free radical scavenging activity, namely the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation, 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radical scavenging ability, and oxygen radical absorption capacity (ORAC) as well as reducing power and bioactivities such as inhibitory activities against the oxidation of LDL cholesterol and DNA strand breakage. In addition, bioinformatics methods were employed to predict bioactive peptides. The research mainly investigated to release of potent antioxidative, angiotensin-converting enzyme (ACE) inhibitory peptides and dipeptidyl peptidase IV (DPP IV) inhibitors using *in silico* approaches. The protein hydrolysates derived from sophia and camelina exhibited superior radical scavenging activity compared to protein isolates, particularly against DPPH and ABTS radicals. Especially in the case of camelina peptide fractions, smaller-size peptides showed significantly higher radical scavenging activity and potency than larger-size peptides. The hydrolysates produced with the Alcalase enzyme demonstrated the highest capacity for scavenging hydroxyl radicals and exhibited excellent oxygen radical absorbance capacity. Additionally, the hydrolysates showed inhibitory effects on LDL cholesterol oxidation and protected against DNA damage caused by hydroxyl and peroxyl radicals. The study successfully employed bioinformatics methods to predict bioactive peptides, revealing that the selected peptides exhibited both ACE and DPP IV inhibitory activities. Importantly, all resistant peptides to digestion were found to be non-toxic. Furthermore, the *in silico* prediction based on physicochemical properties and Lipinski's rule of five suggested that most peptides possess favorable drug-like properties.

These findings indicate that the protein isolates and hydrolysates derived from camelina and sophia seed meal have desirable functional properties and exhibit antioxidant activities. As a result, they are considered valuable sources of bioactive peptides for developing available food ingredients and nutraceutical products. Therefore, camelina/sophia protein hydrolysates show promise as functional food ingredients and nutraceuticals.

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LIST OF ABBREVIATIONS

AL	Alcalase
AA	Amino acid
AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
ABTS	2,2′-Azino-bis-3-ethylbenzthiazoline-6-sulfonate
ACE	Angiotensin-I-converting enzyme
ADME	Absorption, distribution, metabolism, and excretion
ANOVA	Analysis of variance
AE	Alkaline extraction
BPB	Bromophenol blue
CPH	Camelina protein hydrolysates
CPI	Camelina protein isolates
DMPO	5,5-Dimethyl-1-pyrroline-N-oxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Distilled water
DPP IV	Dipeptidyl peptidase IV
EAI	Emulsifying ability index
ESI	Emulsifying stability index
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EPR	Electron paramagnetic resonance
FDA	Food and Drug Administration

FC	Foaming capacity
FS	Foam stability
FL	Flavourzyme
FAO	Food and Agriculture Organization
FRAP	Ferric reducing antioxidant power
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donors
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHL	Hippuryl-L-histidyl-L-leucine
HPLC-MS/MS	High-performance liquid chromatography-mass spectrometry/ mass spectrometry
LC-MS/MS	Liquid chromatography-mass spectrometry / mass spectrometry
LDL	Low-density lipoprotein
LIPO	Lipophilicity
MS	Mass spectrometry
NF	Nanofiltration
OHC	Oil holding capacity
ORAC	Oxygen radical absorbance capacity
PI	Protein isolates
pI	Isoelectric point
ROS	Reactive oxygen species

ROTB	Rotatable bonds
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH	Surface hydrophobicity
SPI	Sophia protein isolates
Soy PI	Soy protein isolates
TE	Trolox equivalents
TEAC	Trolox equivalent antioxidant capacity
TPSA	Topological polar surface area
UAE	Ultrasound-assisted alkali extraction
UF	Ultrafiltration
WHC	Water holding capacity
WHO	World Health Organization

List of amino acids abbreviations

Amino acid	Abbreviation (3 letters)	Abbreviation (1 letter)
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Trytophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

LIST OF PUBLICATIONS

1. Ngo, N.T.T., and Shahidi F. (2021), Functional properties of protein isolates from camelina (*Camelina sativa* (L.) Crantz) and flixweed (sophia, *Descurainis sophia* L.) seed meals. *Food Production, Processing and Nutrition*, *3*, 1-10.

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This work in chapter 5

Notes:

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CHAPTER 1

Introduction and overview

Proteins play a crucial role in both biological systems and human nutrition. They, together with carbohydrates and lipids, are considered as one of three necessary macronutrients. Compared with animal-based protein, natural plant-derived proteins are currently receiving much interest as they are with a sustainable availability and economic attraction (Deng et al., 2019; Du et al., 2018). Hence, the diversified sources of plant proteins increasingly address the worldwide requirement as an ingredient in the food industry. Over the last few years, the attraction of bioactive peptide and proteins from food processing by-products has increased. Furthermore, identification of bioactive peptides encoded in oilseed processing waste has also received much recent attention. It has also been highlighted as a viable biorefinery strategy for addressing the detrimental effects of unregulated waste disposal on the environment (Tavarini et al., 2021; Udenigwe and Aluko, 2012). In this regarding, camelina and sophia seed meals, as a by-product after extraction, have emerged as a potential high protein functional food ingredient with beneficial health effects.

Protein isolates play an integral part of the development of food with desirable functional properties and their hydrolysates have pharmaceutical value (Yagoub et al., 2017). Many efforts have been made to develop efficient methods to produce a high-quality protein with good utilization value (Ambigaipalan et al., 2015; Boyle et al., 2018; Dong et al., 2011). Extraction is the first vital stage for isolating and recovering proteins. Firstly, the traditional extraction method has been applied for extracting plant proteins based on an

aqueous extraction technique with salt, alkali, or an organic solvent. Protein has been separated by isoelectric precipitation and ultrafiltration. Alkaline extraction has become the most popular technique for preparation of protein isolate because of its simplicity and cost effectiveness (Pastuszewska et al., 2000; Phongthai et al., 2016). However, this technique has some potential problems such the presence of complex protein constituents with isoelectric points and a wide range of molecular weight distribution (Rabb et al., 1992; Berot et al., 2005). As a result, a comprehensive extraction technique plays an important role in the development of extracting proteins. Secondly, ultrasound-assisted extraction (UAE) is one of the most successful extraction methods because it brings some potential benefits such as a short extraction time, high extraction yield, low amount of solvent use and the improvement in solubility (Yagoub et al., 2017; Zou et al., 2017). Moreover, compared with the conventional extraction, essential amino acids of the sonicated rapeseed protein isolates were significantly different (Yagoub et al., 2017). Additionally, the sonicated protein extraction increased gelation capacity while decreasing foaming capacity (Ly et al., 2018). Therefore, the functional and nutritional properties of food proteins are improved by using ultrasonic-assisted technology.

Protein hydrolysates are produced by the cleavage of peptide bonds that can be achieved by enzymatic or chemical processes. Alkaline and acid hydrolysis tend to be difficult to control, and yield products with modified amino acids while enzymatic process can be conducted under mild conditions by using specific proteases in order to cleave specific peptide bonds. Therefore, enzymatic modification of proteins is widely used in the food industry (Mullally et al., 1994). Traditional methods have been employed to discover

and create novel bioactive peptides, including numerous processes for the separation, purification, and identification of bioactive peptides. However, this method is time-consuming and laborious. Currently, these problems have been solved by using bioinformatic methods for predicting and discovering potent peptides derived from food proteins that save time and work better than traditional methods (Agyei et al., 2018; Panjaitan et al., 2018; Udenigwe, 2014). It is important to note that bioinformatic predictions are often combined with experimental validation to confirm the potency of predicted peptides. The integration of computational methods with experimental techniques can significantly accelerate the discovery and optimization of potent peptides for various applications, such as drug development, antimicrobial agents, and peptide-based therapeutics. Many studies have reported the functional properties and antioxidant activities of protein hydrolysates from plant sources such as rapeseed, hemp seed, buckwheat, corn, and date seed proteins have used hydrolysates to prepare bioactive peptides (Ambigaipalan and Shahidi, 2015; Ambigaipalan and Shahidi, 2017; Cumby et al., 2008; Girgih et al., 2013; Tang et al., 2009; Zhou et al., 2012). Research suggests that plant protein hydrolysates may offer various health benefits, including improved blood pressure regulation, cholesterol-lowering effects, and potential anti-cancer properties (Hertzler et al., 2020). Recent attention has focused on discovering bioactive peptides from oilseed processing discards, highlighting their potential as a biorefinery strategy to address environmental issues caused by unregulated waste disposal. Notably, camelina and sophia seed meals are distinguished by their high protein content among various oilseed varieties. In contrast, there are a few studies of extracted protein and hydrolysates from camelina (Boyle et al., 2018; Li et al., 2014). Additionally, studies have investigated to the fatty acid

profile and phenolic compounds of sophia seeds (Hadinezhad et a., 2015; Rahman et al., 2018). Based on the available literature, very limited of studies have been carried out to determine proteins in camelina and sophia seed meals, and their functional properties and antioxidant activities. Limited number of studies have reported the use of camelina meals as precursor for bioactive peptides. So far, no studies have been carried out to explore drug likeliness of camelina and sophia meals derived bioactive peptides. Hence, more studies are needed to fully explore, understand, and validate these potential benefits. In this study focused on exploring the potential of camelina and sophia meal proteins as viable sources of bioactive peptides and food ingredients for human consumption, the following aim and objectives were undertaken.

Aim and objectives: The primary objective of this research was to assess the bioactive and technical properties of camelina and sophia protein isolates and their hydrolysates. The goal was to identify and potentially utilize one or more of these components as functional or nutraceutical ingredients in food systems.

The main objectives of the research were as follows:

1. To determine the effects of the conventional extraction and ultrasonic-assisted extraction on the functional properties of camelina protein isolates and sophia protein isolates.

2. To investigate the impact on bioactive activities and biological systems of the sequential and individual application of specific endopeptidases and exopeptidases in the preparation of protein hydrolysates and their fractions from camelina and sophia protein isolates.

3. To employ a bioinformatics-integrated approach to predict the potential bioactive peptides and assess the physicochemical properties of peptides derived from camelina and sophia protein hydrolysates.

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CHAPTER 2

Literature review

2.1 The role of plant proteins as functional ingredients in food systems

Proteins are an essential macronutrient in our diet, serving as vital sources of energy and supplying the necessary amino acids our bodies require. These amino acids are instrumental in promoting healthy growth and facilitating the diverse functions of our bodies. In the food system, plant proteins hold significant importance as functional ingredients, providing a wide array of advantages from both nutritional and sustainability perspectives (Hertzler et al., 2020).

Plant-based proteins serve as a viable alternative to animal proteins in the realm of food. They offer a wide range of options for individuals seeking alternative protein sources, whether due to dietary preferences, ethical considerations, or health concerns. Legumes, cereals, and oilseeds are among the many plant-based sources that provide ample protein content. These plant proteins not only contribute to a well-rounded diet but also offer various health benefits, such as being generally lower in saturated fats and cholesterol compared to animal proteins. Furthermore, incorporating plant-based proteins into our diets can have positive environmental implications, as they typically require fewer resources and generates fewer greenhouse gas emissions compared to animal protein production (Langyan et al., 2021). Besides, with the growing global population, plant proteins can contribute to addressing food security challenges. Plant-based protein sources can be produced more efficiently and sustainably, potentially reducing reliance on resource-intensive animal agriculture. Furthermore, plant proteins possess various functional

properties that make them valuable ingredients in food manufacturing. They can act as emulsifiers, stabilizers, thickeners, foaming agents, gelling agents, and texturizers. These properties enhance the texture, mouthfeel, and stability of food products (Deng et al., 2019; Dong et al., 2011; Zhang et al., 2018). Plant proteins exhibit versatility in food applications, allowing their incorporation into a wide range of products. They can be used in meat alternatives, dairy alternatives, baked goods, snacks, beverages, and more. Plant-based protein powders, such as pea, soy, or hemp, are popular additions to smoothies and shakes (Kyriakopoulou et al., 2021).

Plant proteins have been associated with various health benefits. They can contribute to weight management, cardiovascular health, blood sugar control, and reducing the risk of chronic diseases such as type 2 diabetes and certain types of cancers (Samtiya et al., 2021; Udenigwe and Aluko, 2012). Overall, plant proteins provide a wide range of nutritional, sustainability, and functional benefits in the food system. Their integration into food products promotes a more balanced and environmentally friendly approach, aligning with consumers' evolving preferences and needs. It is worth emphasizing that a well-rounded diet that includes diverse protein sources, encompassing both plant and animal proteins, can supply all the essential amino acids and vital nutrients required for optimal health. Nevertheless, increasing the inclusion of plant proteins in one's diet can yield multiple advantages and contribute to a healthy lifestyle.

2.2 Camelina as a novel plant protein

Camelina is a genus in the Brassicaceae (mustard) family. The most cultivated species is camelina sativa, also known as false flax, gold-of-pleasure, or wild flax. It is

planted as a native plant in Northern Europe and Central Asia. Camelina is a hardy plant, tolerant of poor soils and cooler climates, which makes it suitable for cultivation in many regions. Camelina is a new potential oilseed source in North America and a novel source of plant protein. Camelina meal is the by-product of the oil extraction process from Camelina seeds and is utilized for animal feed, including aquaculture. Defatted camelina meal is rich in proteins (45%) and contains approximately residual oil (10%), insoluble fiber (15%), soluble carbohydrates (10%), minerals (5%), and a mixture of phytochemical components consisting primarily of glucosinolates (GSLs), flavonoids, and other components (Das et al., 2014). GSLs are present as secondary metabolites in the Brassicaceae and act as natural pesticides, preventing herbivory (Halkier and Gershenzon, 2006). The hydrolysis of 2-hydroxy-3butenyl glucosinolate forms an oxazolidine-2-thione, which causes goiter and brings some harmful impact on animal nutrition (Halkier and Gershenzon, 2006). However, defatted camelina meal contains two majors aliphatic glucosinolates, glucoarabin (GS9) and glucocamelinin (GS10), with traces of homoglucocamelinin (GS11) (Das et al., 2014). They contain terminal methylsulfinyl groups with various length aliphatic chains connecting to the glucosinolate functional group, similar to that of glucoraphanin present in broccoli and other crucifera vegetables. Some studies have revealed that the degradation product of glucoraphanin acts as a potent anticancer compound (Fahey et al., 2001; Shapiro et al., 2001). Furthermore, the nutritional quality of camelina protein is similar to that of canola protein which possibly competes with soy protein for some applications targeting the use of plant proteins (Li et al., 2015). Protein isolates and hydrolysates derived from camelina could serve as a valuable plant-based protein resource for human consumption. Nevertheless, studies focusing on the

extraction and benefits of these camelina proteins and hydrolysates remain limited (Boyle et al., 2018; Li et al., 2014). Therefore, further investigation is necessary to augment the functional properties and antioxidant activities of these extracted camelina proteins, thereby broadening their potential for food applications.

Table 2.1 Amino acid profile of protein from camelina meal

Essential AA	g/100g protein	Nonessential AA	g/100g protein
Leucine	6.91	Glutamic acid	14.98
Valine	6.04	Aspartic acid	9.03
Phenylalanine	5.21	Glycine	6.07
Isoleucine	4.64	Proline	6.02
Lysine	4.49	Alanine	5.98
Threonine	2.88	Serine	5.96
Methionine	2.48	Tyrosine	3.64
Trytophan	1.28		
Arginine	8.27		
Histidine	4.21		

2.3 Sophia as a novel plant protein

Sophia (*Descurainis sophia* L.), known as Flixweed and a member of the Brassicaceae family, is found not only throughout Canada but also in countries like Iran

and China. Sophia seeds have been used as a traditional medicine to relieve cough and chest discomfort, prevent asthma and treat cancer (Hadinezhad et al., 2015; Khan and Wang, 2012). The seeds of sophia are known to contain a significant amount of protein and oil around 28% and 33 %, respectively (Rahman et al., 2018). The amino acids in sophia seed meals include aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, and arginine (Mohamed and Mahrous, 2009). Research has delved into the fatty acid profile and phenolic compounds found in sophia seeds (Hadinezhad et al., 2015; Rahman et al., 2018). However, despite the availability of information about this plant, there seems to be a gap in the literature with regards to the analysis of proteins in sophia meal seeds, particularly their functional properties and antioxidant activities.

Table 2.2 Amino acid profile of protein from sophia seeds

Essential AA	**g/100g protein**	**Nonessential AA**	**g/100g protein**
Threonine	5.5	Aspartic acid	11.3
Valine	8.5	Serine	5.3
Isoleucine	5.8	Glutamic acid	13.6
Leucine	8.9	Proline	27.2
Phenylalanine	6.9	Glycine	5.8
Lysine	7.8	Alanine	7.3
Histidine	4.6	Tyrosine	2.1
Arginine	4.6		

2.4 Approaches for isolating proteins

Multiple methods are employed to produce protein concentrates and isolates from plants sources. These techniques include isoelectric precipitation, ultrafiltration, air classification, pin milling, and wet extraction. Among these methods, the pH-shift protein isolation method, also called the acid or alkaline solubilization and precipitation method, or isoelectric solubilization/precipitation methods stand out as the most utilized due to its advantages of higher protein purity and simplicity (Boye et al., 2010). The pH-shift method involves subjecting a protein molecule to a pH level significantly lower or higher than its isoelectric point (pI). This manipulation of pH leads to the protonation or deprotonation of specific amino or carboxyl groups within the protein molecule, resulting in the formation of a highly positively or negatively charged structure, respectively. Indeed, the stronger charge repulsion that arises from the extreme pH conditions in the pH-shift method promotes the dissociation of polypeptide segments within the protein molecule. This charge repulsion weakens the electrostatic interactions and stabilizing forces that maintain the protein's folded structure. As a result, the protein undergoes structural unfolding or partial unfolding, where the native conformation of the protein is disrupted. This structural unfolding allows for greater accessibility of the protein's surface and can facilitate subsequent interactions or modifications during the isolation and purification process (Jiang et al., 2009). When a structurally unfolded or partially unfolded molecule is exposed to a neutral pH condition, where intramolecular charge repulsions are significantly reduced, refolding of the molecule takes place. This refolding process leads to the formation of a structure with molten globule characteristics. The resulting structure displays significantly

enhanced surface hydrophobicity and a balanced distribution of hydrophobic and hydrophilic regions, resulting in improved surface properties (Figure 2.1). In this pH-shift method, the proteins from plant tissues undergo precipitation by shifting the pH from acidic to alkaline conditions (pH 1.5 to pH 12). The subsequent use of isoelectric precipitation allows for the separation and recovery of proteins based on their different isoelectric points (Chavan et al., 2001; Dong et al., 2011; Ngo and Shahidi, 2021). Currently, the pH-shift method has been applied to isolate proteins from various plant seeds. By manipulating the pH, seed proteins can be precipitated and recovered, enabling their further study and potential application in food or industrial processes. For examples, the pH-shift method has been applied to extract storage proteins from beach pea (Chavan et al., 2001), cashew nut (Ogunwolu et al., 2009), rapeseed (Dong et al., 2011), chickpea (Tontul et al., 2018), mung bean (Du et al., 2018), and Chinese quince seed (Deng et al., 2019).

Ultrasound-assisted extraction (UAE) has gained significant attention in the past decade as a method for extracting proteins from various plant seeds. Ultrasound technology has found applications in the food industry to modify the characteristics of food components in liquid media. By utilizing high-intensity, low-frequency ultrasound (16-100 kHz), mechanical acoustic waves are employed to generate cycles of compression and rarefaction within the liquid. During the compression cycles, the small gas bubbles present in the liquid experience growth due to the alternating pressure fluctuations. Eventually, these bubbles reach a critical size and collapse, resulting in cavitation. Cavitation leads to the localized accumulation of energy, causing rapid and brief heating up to temperatures and an increase in pressure (Knorr et al., 2004; Silventoinen and Sozer, 2020). Beyond the

thermal and pressure effects, cavitation also triggers other phenomena that contribute to modifying the properties of the treated liquid media. These include mixing, microstreaming currents, shear stresses, and turbulence. Such effects facilitate enhanced mass transfer, improved dispersion, emulsification, particle size reduction, and other beneficial alterations in the food components. An important advantage of ultrasound technology is its relatively low energy consumption and reduced environmental impact when compared to certain conventional food processing methods. Utilizing ultrasound can minimize reliance on high-temperature processes or chemical additives, thereby promoting sustainability. Additionally, ultrasound processing can be carried out at ambient or mild temperatures, preserving the nutritional quality and sensory attributes of food products (Fatima et al., 2023; Ly et al., 2018; Yagoub et al., 2017; Zou et al., 2017). Previous studies have demonstrated that ultrasound treatment has positive effects on the properties of proteins derived from various sources. For examples, it has been found to improve the water solubility of proteins from soy (Hu et al., 2013), black bean (Jiang et al., 2014), pea (Jiang et al., 2017), and canola (Flores-Jimenez et al., 2019). This enhancement in water solubility is advantageous as it increases the dispersibility and functional capabilities of these proteins in aqueous systems. Besides, it has been observed to alter the surface hydrophobicity of soy and pea protein isolates, as well as the zeta-potential of black bean protein isolate. These changes can influence protein interactions and stability in various applications. Furthermore, ultrasonication has been found to enhance the foaming properties of canola and pea protein isolates (Flores-Jimenez et al., 2019; Xiong et al., 2018), and it has also demonstrated improvements in the oil absorption capacity, emulsifying properties, and gelation properties of canola protein isolate.

In summary, ultrasound treatment has proven to be a valuable technique for modifying the properties of proteins. It enhances water solubility, reduces particle size, modifies surface characteristics, improves foaming properties, and enhances functionalities such as oil absorption, emulsification, and gelation. These findings have significant implications for the development of protein-based ingredients and food products.

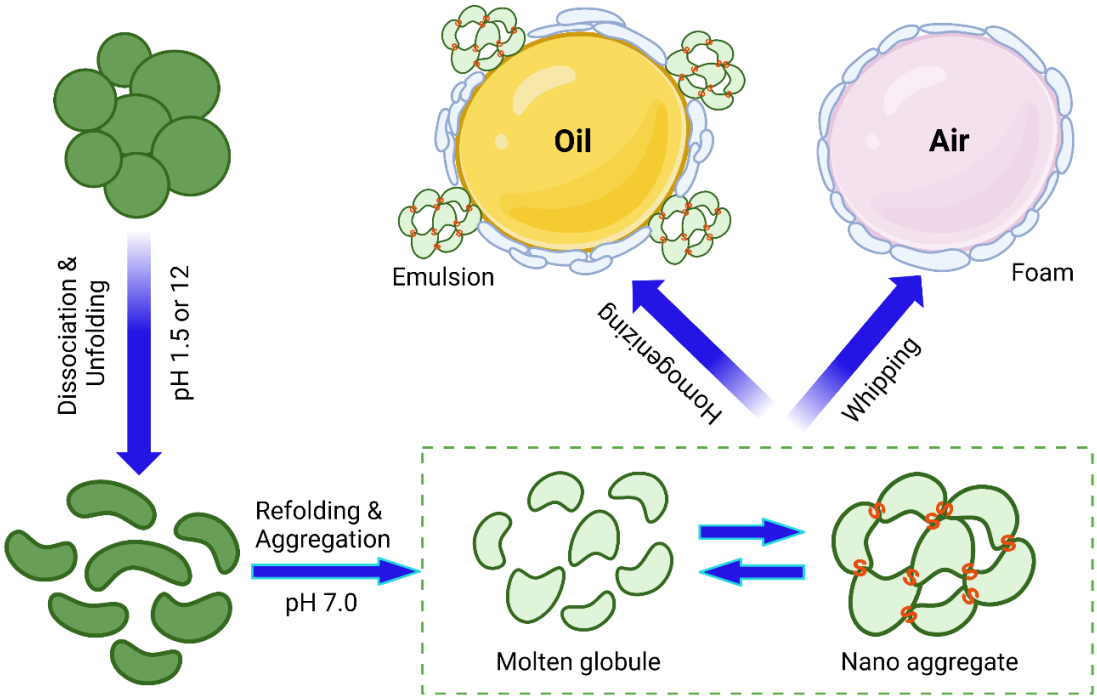

Figure 2.1 The pH shift process and its possible impact on the surface properties of proteins adapted from Jiang et al., 2018.

2.5 Functionality of food proteins

Functional properties of proteins in the context of food refer to the specific characteristics that determine their usefulness and applications in food products. The

physicochemical properties of proteins, in addition to their nutritional relevance, play a crucial role in determining their functionalities in food systems. The concentration of proteins, as well as the processing methods used and environmental conditions, can significantly influence their functional roles in food. Hence, the selection of protein types for specific functionalities also considers factors such as taste, nutritional profile, allergenicity, and cost. Food scientists and technologists carefully choose proteins based on their structural properties and functional attributes to achieve the desired characteristics in the final food product (Alashi et al., 2013; Foegeding and Davis, 2011). Food proteins serve various important functions that significantly influence the desired textural characteristics of food. These functions include solubility, emulsification, gelation, foaming, water binding, and heat stability.

2.5.1 Solubility

The solubility of proteins is a critical physicochemical and functional property due to its wide variation across pH levels, temperature range, nitrogen concentrations, and ionic conditions. This characteristic holds significant importance as it impacts the behavior and functionality of proteins in diverse applications. Soluble proteins are crucial for achieving a homogeneous distribution of molecules in colloidal systems, thus improving interfacial properties. The decrease in solubility is linked to protein denaturation and aggregation, leading to unattractive appearance and undesirable mouthfeel. The degree of protein insolubility serves as an indicator of denaturation and loss of solubility, affecting functionalities such as foaming, emulsification, and gelation (Deng et al., 2019; Tontul et al., 2018). Solubility is determined by the equilibrium between repulsive electrostatic and

attractive hydrophobic forces. Hydrophobic interactions, including protein-protein interactions, reduce solubility and promote protein aggregation. Ionic residues on the protein surface contribute to electronic repulsions, enhancing solubility. Higher ionic interactions and lower hydrophobicity result in increased protein solubility (Alashi et al., 2013). The solubilization of proteins can also be affected by treatments such as heat, alkali, and acidic modifications, which alter protein configuration. The ionic strength of the solution is another factor influencing protein solubility, with low ionic strength promoting solubility and high ionic strength leading to protein precipitation. Neutral salt solutions can increase solubility through the salting-in effect, while high salt concentrations can cause protein aggregation and precipitation through the salting-out effect (Duong-Ly and Gabelli, 2014; Kramer et al., 2012).

2.5.2 Water (WHC)- and oil-holding capacity (OHC)

Proteins possess important functional properties such as water-holding capacity (WHC) and oil-holding capacity (OHC) that have a significant impact on the texture, viscosity, and dehydration processes during food storage. The amount of water associated with proteins is influenced by various factors, including the amino acid profile, charged residues, conformation, and hydrophobicity of the protein. Proteins with a higher number of charged residues tend to have a greater capacity to bind water. Temperature, pH, ionic strength, and protein concentration also affect the amount of water associated with proteins (Alashi et al., 2013; Fatima et al., 2023).

Water holding capacity refers to a protein's ability to retain water within its structure. Proteins with high WHC efficiently bind water molecules, which is beneficial for

food products. When proteins with high WHC are used in food formulations, they help preserve moisture and prevent dehydration during storage. This is crucial for maintaining the quality and shelf life of food, as dehydration can lead to undesirable changes like texture alteration, flavor loss, and overall quality deterioration. Furthermore, proteins with high WHC can also dehydrate other ingredients in the food, making the product less sensitive to changes in storage humidity. By effectively binding water, proteins create a stable environment within the food, minimizing the risk of moisture migration and ensuring the desired texture and consistency (Zayas, 1997; Zou et al., 2017).

Oil holding capacity refers to a protein's ability to bind and absorb oil. Proteins with high OHC are valuable in various food applications. For example, in emulsion-based products like salad dressings or mayonnaise, proteins with high OHC contribute to the stability of the emulsion by binding to oil droplets and preventing separation. This enhances the desired texture and stability of the product (Metri-Ojeda et al., 2022). Previous studies have demonstrated that the process of defatting oil seed meals leads to an increase in protein solubility, as well as the water and oil absorption capacities. These studies have also revealed significant variations in the water absorption capacity and oil absorption among different oil seed meals, including flax meal, soy meal, canola meal, and camelina meal (Boyle et al., 2018; Khattab and Arntfield, 2009). However, there is limited publication on the WHC and OHC of intact camelina and sophia meal proteins extracted from the meal. There is limited published research available specifically on the WHC and OHC of intact camelina and sophia meal proteins extracted from the meal. The scientific literature on

these specific protein sources might be scarce or relatively limited compared to more extensively studied proteins.

2.5.3 Emulsifying properties

An emulsion refers to a mixture where one liquid phase is suspended within another, despite their immiscibility. In this arrangement, one of the phases forms distinct droplets that are dispersed throughout the continuous phase. Acting as a barrier between the two phases, there exists an interfacial layer occupied by essential surfactant or emulsifier substances. These compounds can bind (adsorb) to the lipid phase of the oil-water interface, contributing to the stability of the emulsion (Mcclements, 2016). There are three commonly encountered types of emulsions: water-in-oil (W/O) emulsion, oil-in-water (O/W) emulsion, and multiple emulsion. Multiple emulsion, also known as a complex emulsion, is a specialized type of emulsion characterized by soft materials composed of dispersed droplets that contain smaller droplets within them. Double emulsions, a subtype of multiple emulsions, include oil-in-water-in-oil (O/W/O) emulsions and water-in-oil-in-water (W/O/W) emulsions. Each type has a distinct composition, with water and oil phases arranged in a specific manner (Figure 2.2) (He et al., 2015; Tian et al., 2022).

Proteins differ from small-molecular-weight surfactants in their role as emulsifiers due to their unique molecular structures. The specific protein present in a food emulsion determines its distinctive properties. Proteins function as emulsifying agents because of their large molecular weights and simultaneous hydrophilic and hydrophobic properties. This allows proteins to adsorb at the interface between oil and water, reducing the interfacial tension and facilitating the formation and stabilization of emulsion droplets.

Consequently, less mechanical energy is needed to achieve a desired droplet size in the emulsion. The strength, compactness, elasticity, and electrical properties of the interfacial film surrounding the emulsion droplets are also important for emulsion stability and are influenced by the proteins present. These properties of the interfacial film can have an impact on the long-term stability of the emulsion (Dalgleish, 1997; Alashi et al., 2013). The emulsifying properties of proteins are influenced by various molecular properties, including molecular mass, hydrophobicity, conformation stability, charge, and physicochemical factors such as pH, ionic strength, and temperature. Proteins with appropriate molecular characteristics can effectively act as emulsifiers in specific conditions. It is important to understand these factors to utilize proteins as emulsifiers effectively in different food applications. For example, the solubility of proteins is an important factor in their effectiveness as emulsifiers. Highly insoluble proteins are not suitable emulsifiers and can lead to the coalescence of emulsion droplets.

Indeed, the emulsifying properties of proteins are commonly evaluated using three main methods: emulsifying capacity, emulsion stability, and emulsifying activity. Emulsifying capacity of a protein by measuring the volume of oil that can be emulsified before the emulsion undergoes phase inversion or collapses. It provides information on the maximum amount of oil that a protein can stably emulsify. Emulsion stability refers to the ability of a protein to form and maintain an emulsion without significant changes over a specific duration and under specific conditions. This method assesses the resistance of the emulsion to creaming, coalescence, or phase separation, providing insights into the long-term stability of the emulsion. The emulsifying activity index measures the efficiency of a

protein in stabilizing the oil-water interface. It is calculated as the area of the interface stabilized per unit weight of the protein and is typically expressed in m^2/g. The higher the EAI value, the better the protein's ability to stabilize the interface and form a stable emulsion (Moure et al., 2006).

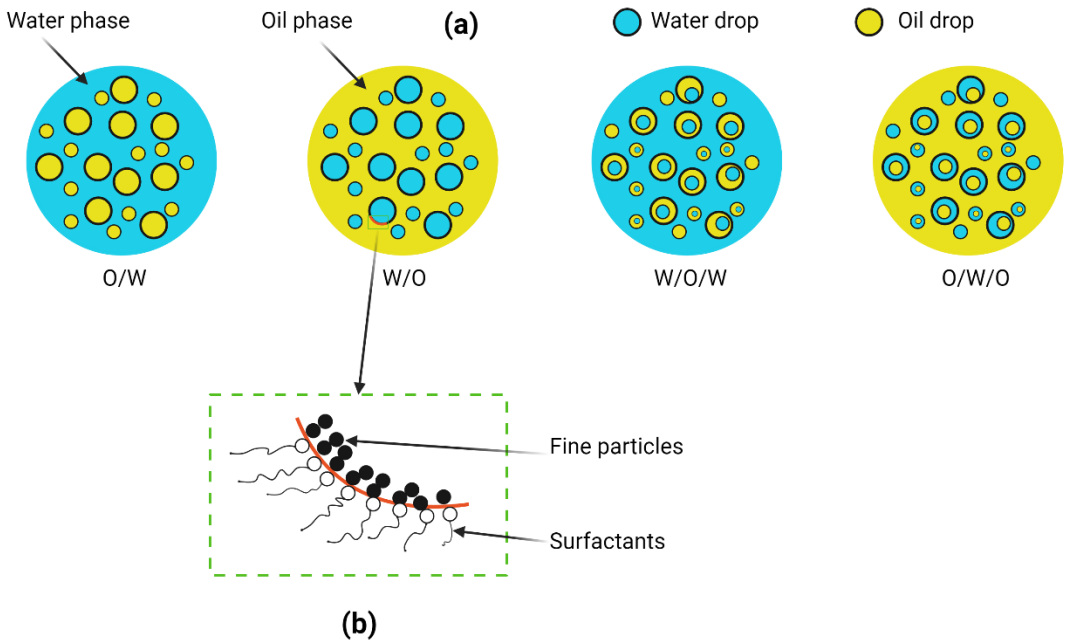

Figure 2.2 Schematic of common types of emulsions and their compositions adapted from He et al., 2015. (a) Emulsions generated in crude oil production and transportation, and (b) Formation of rigid films at the oil–water interface.

2.5.4 Foaming properties

Proteins serve as the primary surfactants necessary for stabilizing the dispersion of gas within a liquid phase in food products. The creation of foam necessitates a substantial

interface area that allows air to be integrated into the liquid phase and forms a barrier that can resist both internal and external pressures. Indeed, a wide range of processed foods including whipped cream, ice cream, cakes, and marshmallows are examples of foam-based products (Alashi et al., 2013). In the foaming process, the solubility and interfacial characteristics of proteins significantly influence the behavior of the protein-based foam. Owing to their amphiphilic nature, proteins aid in reducing both the surface and interfacial tension of the liquid. This reduction in surface tension is accomplished through the orientation of proteins as they unfold at the interface. During this unfolding, proteins arrange themselves such that their hydrophobic (water-repelling) components extend into the air, while their hydrophilic (water-attracting) components orient toward the aqueous phase. This configuration allows for the stable formation of a foam structure. The capacity of a foam is defined by the protein's ability to lower the surface tension, its molecular adaptability, and its physicochemical attributes such as its hydrophobicity, net electrical charge, the distribution of that charge, and its hydrodynamic characteristics (Moure et al., 2006).

In food manufacturing, the capacity to form and stabilize foams can be improved by enhancing certain properties of proteins, such as their solubility, surface flexibility, and the distribution of their charge. This results in a better dispersion of proteins at the interface between air and water, thus increasing the protein's ability to absorb air. Moreover, maintaining a pH level above the protein's isoelectric point increases the protein's capacity to encapsulate air during the foaming process. Therefore, the pH level of the medium in which dispersion takes place is key in boosting the foam forming ability of food proteins.

Lastly, amplifying repulsion forces and ensuring the availability of hydrophobic amino acids can prevent air bubbles from merging, ultimately leading to more stable foam (Deng et al., 2019; Tontul et al., 2018). The formation of foam is typically assessed by considering two main factors: the foaming capacity and the foaming stability of a protein. Foaming capacity refers to the volume ratio of the produced foam to the original volume of the solution, while foaming stability denotes the duration for which the foam can maintain its structure. The viscous, elastic, cohesive, gas-impermeable, and continuous film that forms around air bubbles significantly contributes to the stability of a foam (Deng et al., 2019). These properties depend on factors such as the type and concentration of the proteins or other surfactants used, as well as the pH, ionic strength, and temperature of the liquid phase. Adjusting these factors can help optimize the stability of the foam.

2.6 Hydrolysates and bioactive peptides: Production and characterization

Proteins are indeed food macronutrients that play a crucial role in providing energy and supplying the body with essential amino acids necessary for various biological processes, including growth and maintenance. Proteins can also serve as functional ingredients and therapeutic agents due to the presence of bioactive peptides within their structure. Bioactive peptides are short chains of amino acids encrypted within the protein molecules that exhibit specific biological activities and health benefits when consumed. These peptides have been extensively studied and have been found to possess a wide range of bioactivities, including anti-adhesive, anti-cancer, anti-diabetic, lipid-lowering, immunomodulatory, anti-inflammatory, antioxidative, and antihypertensive properties (Samtiya et al., 2021; Mora et al., 2018). These bioactivities are attributed to the specific

amino acid composition and sequence of the peptides. Some food-derived bioactive peptides share structural similarities with endogenous peptides found in the human body, allowing them to interact with specific receptors and elicit physiological responses. The growing interest in food-derived bioactive peptides stems from their diverse range of functionalities and potential health benefits. Researchers continue to explore and identify new bioactive peptides in various food sources, with the aim of developing functional foods, nutraceuticals, and therapeutic applications to improve human health and well-being.

Several food sources, including animals, plants, and marine products, have been used as sources of bioactive peptides. Production of such products is generally carried out in pursuit of value-added use of underutilized proteins or protein-rich food industry's by-products, and utilization of proteins containing specific peptide sequences or amino acid residues of pharmacological interest (Udenigwe and Aluko, 2012). There are multiple methods available to produce peptides with biological activity including chemical hydrolysis, enzymatic hydrolysis, and microbial/fermentation foods. These methods have been widely utilized to produce bioactive peptides from various protein sources. By employing these techniques, it is possible to generate peptides with diverse biological activities, such as antioxidant, antimicrobial, anti-inflammatory, and ACE-inhibitory properties. To date, using enzymes to produce protein hydrolysates has gained much attention as a potential method of making functional food ingredients and nutraceuticals for disease prevention and health promotion (Alashi et al., 2014; Ambigaipalan et al., 2015; Senadheera et al., 2021). Generally, an acceptable food protein source must be chosen

based on the physiological properties of products and then treated with a single or multiple specific or nonspecific protease to release peptides of interest. The type of enzyme determines the cleavage pattern of peptide bonds. The principal enzymes employed for peptide production are endo- and exo-proteinases such as trypsin, chymotrypsin, and pepsin (endoproteinases), as well as commercial proteases such as Alcalase and Flavourzyme (Kristinsson and Rasco, 2000; Udenigwe and Aluko, 2012).

2.6.1 The role of enzymes in the production of hydrolysates and peptides

Enzymes are biological catalysts that increase the rate of chemical reactions in living organisms. They play a crucial role in various physiological processes by facilitating the conversion of substrates into products. Enzymes themselves remain unchanged after the reaction and can be reused. The enzymatic hydrolysis of proteins refers to their breakdown into smaller peptide fragments or individual amino acids through the action of proteases. Compared to chemical hydrolysis, enzymatic hydrolysis applies milder reactions, and offer more predictions of products (Udenigwe and Aluko, 2012; Tavano et al., 2018). Hence, enzymatic hydrolysis is commonly conducted in food biotechnology (Figure 2.3).

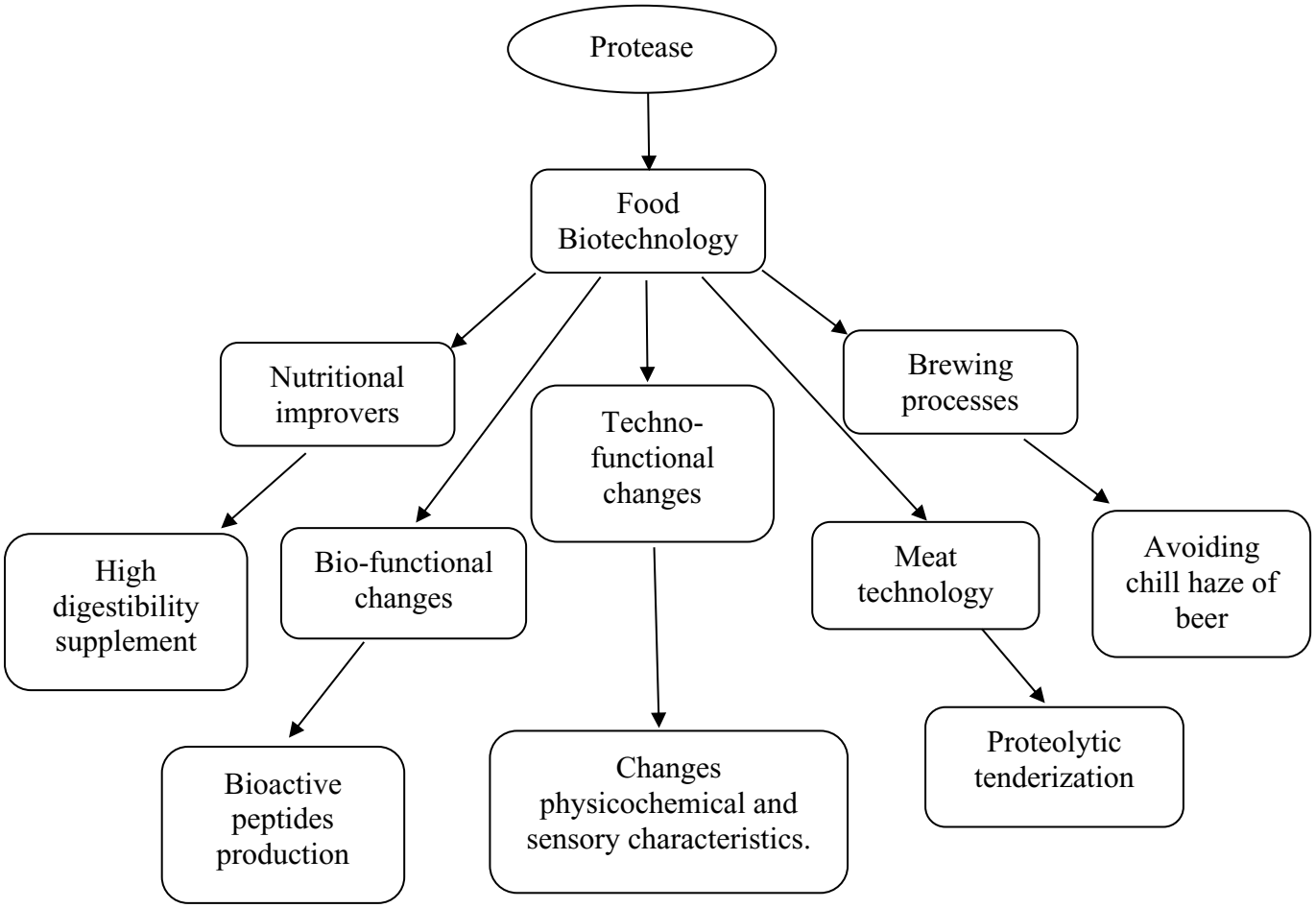

Figure 2.3 Potential utilizations of proteases in food biotechnology adapted from Tavano et al. (2018)

Proteinases, also known as proteases or peptidases, are enzymes that specifically catalyze the hydrolysis of peptide bonds in proteins. They can be classified based on the chemical nature of their active site residues such neutral proteinases (trypsin, chymotrypsin), and acidic proteinases (pepsin, renin) (Alashi et al., 2013). Furthermore, proteinases can be differentiated based on their function as either endopeptidases or exopeptidases. These distinct types of proteinases, including both endo- and exo-proteinases, are employed to facilitate the hydrolysis of peptide linkages in various biological processes. Endopeptidases are enzymes that catalyze the hydrolysis of peptide bonds between amino acid residues within polypeptide chains. They target internal peptide bonds, cleaving the chain into smaller fragments. On the other hand, exopeptidases, such as aminopeptidases and carboxypeptidases, are responsible for cleaving the terminal peptide bond of a polypeptide chain or protein (Tavano, 2013; Tavano et al., 2018).

Oilseed meals, also known as press cakes, are the by-products obtained after oil extraction from oilseeds. These meals are commonly used as animal feed due to their high protein content and nutritional value. Recent studies have examined the potential of various oilseed by-products, including canola, flaxseed, sesame, sunflower, pumpkin and hemp seeds, as viable protein sources for human consumption (Alashi et al., 2014; Wei et al., 2018, Chatterjee et al., 2015; Dabbour et al., 2019; Sitohy et al., 2020; Gao et al., 2021). To make oilseed meals suitable for human consumption, further processing and refinement may be necessary. Enzymes can be employed in the processing of oilseed by-products to enhance their value, increase utilization, and reduce waste. A variety of proteinases derived from plants, animals, and microorganisms are employed for the hydrolysis of food proteins.

These proteinases, considered food-grade enzymes, play a crucial role in food processing including Alcalase, Flavourzyme, Protamex, papain, trypsin, chymotrypsin, pancreatin, and Neutrase (Table 2.3). Among these enzymes, Alcalase, a nonspecific endo-protease derived from Bacillus licheniformis, finds numerous applications in various industries. The utilization of Alcalase in the production of protein hydrolysates from date seed, canola, sunflower, hemp seed and other similar protein sources allows for the generation of ingredients that can be used in food products as additives or nutritional supplements (Cumby et al., 2008; Ambigaipalan et al., 2015; Dabbour et al., 2019; Malomo et al., 2014). The resulting hydrolysates may exhibit improved solubility, enhanced flavor, increased digestibility, and potential bioactive properties. For example, the solubility of proteins relies on their hydrophilicity and electrostatic repulsions. When proteins are hydrolyzed, their solubility often increases due to a higher concentration of small peptides, which leads to an accompanying rise in the levels of ionizable amino and carboxyl groups (Tavano, 2013; Ambigaipalan and Shahidi, 2015). Previous studies have reported that enzymatic hydrolysis of isolated peanut protein using Alcalase resulted in improved protein solubility, which was closely associated with a reduction in surface hydrophobicity (Zhao et al., 2011). According to the reported study, the use of rapeseed protein hydrolysates prepared with Alcalase and Flavourzyme resulted in an augmented water-holding capacity in a ground pork meat model system (Cumby et al., 2008). In addition, peptides released from sunflower seed protein after hydrolyzing by Flavourzyme can contribute to the umami flavor (Bao et al., 2020). It is important to note that the specific characteristics of protein hydrolysates, such as the peptide profile and degree of hydrolysis, can be influenced by factors such as the substrate used, hydrolysis conditions (including enzyme concentration,

reaction time, pH, and temperature), and subsequent processing steps (Alashi et al., 2013; Ambigaipalan and Shahidi, 2015; Ngo and Shahidi, 2021). Thus, optimization of these parameters is crucial in obtaining protein hydrolysates with desired functionalities for specific food applications.

Table 2.3 Application of protein hydrolysis enzymes for various oilseed meals

Protein source	Enzyme treatment	Reference
Canola meal (*Brassica napus*)	Alcalase	Zhang et al., 2007
	Alcalase, Flavourzyme	Cumby et al., 2008
	Alcalase, Pepsin, Chymotrypsin, Trypsin, Pancreatin	Alashi et al., 2014
	Pepsin - Pancreatin	Bermejo-Cruz et al., 2023
Peanut meal (*Arachis hypogaea* L.)	Alcalase, Pepsin-Pancreatin	Quist et al., 2009
	Alcalase, Nutrase, Protamex	Jiang et al., 2010
	Alcalase	Zhang et al., 2016
Sesame meal (*Sesamum indicum* L.)	Alcalase, Flavourzyme, Protamex, Neutrase	Kanu et al., 2009
	Metallo-endopeptidase (Protease A Amano 2G)	Das et al., 2012
	Pepsin, Papain, Alcalase	Chatterjee et al., 2015
Pumpkin meal (*Cucurbita pepo* L.)	Alcalase, Flavourzyme	Vastag et al., 2011
	Papain	Sitohy et al., 2020
Sunflower meal (*Helianthus annuus* L.)	Alcalase, Flavourzyme	Megias et al., 2009
	Alcalase	Dabbour et al., 2019
Flaxseed meal (*Linum usitatissimum* L.)	Papain, Trypsin, Pancreatin, Alcalase, Flavourzyme	Karamac et al., 2016
	Alcalase and Flavourzyme	Wei et al., 2018
Hemp seed meal (*Cannabis sativa* L.)	Pepsin - Pancreatin	Girgih et al., 2011
	Pepsin, Pancreatin, Papain, Alcalase	Malomo et al., 2015
	Protamex	Gao et al., 2021

Bioactive peptides are small protein fragments that are inactive within the parent molecule and are activated through enzymatic hydrolysis. This process can occur during digestion or food processing. Digestive proteolysis and bacterial fermentation can release different bioactive peptides (Udenigwe and Aluko, 2012; Alashi et al., 2013). Researchers have shown particular interest in enzymes such as Alcalase, Flavourzyme, Neutrase, and Protamex for their role in protein hydrolysis and their potential to produce bioactive peptides with physiological functions. These enzymes have garnered attention in various food applications due to their ability to selectively break down proteins and generate peptides that offer specific health benefits (Cumby et al., 2008; Jiang et al., 2010; Wei et al., 2018; Gao et al., 2021). These peptides, due to their unique amino acid composition and sequence, exhibit beneficial health effects on the cardiovascular, digestive, immune, and nervous systems including anti-adhesive, anti-cancer, anti-diabetic, lipid-lowering, immunomodulatory, anti-inflammatory, antioxidative, and antihypertensive properties (Tavano, 2013; Tavano et al., 2018; Samtiya et al., 2021). However, when bioactive peptides are directly added to foods, they need to survive the digestive process and avoid degradation in the intestine. Many bioactive peptides show resistance to enzymatic hydrolysis, allowing them to survive digestion to some extent. Peptides generated through *in vitro* digestion may differ from those produced *in vivo* (Tavano et al., 2018).

In short, bioactive peptides are released through enzymatic hydrolysis and have the potential to affect various physiological processes. They are being explored for applications in the food and pharmaceutical industries. Incorporating them into food products requires

considering their survival through digestion and potential differences between *in vivo* and *in vitro* digestion.

2.6.2 Production and purification of bioactive peptides

To comprehend the physicochemical properties and evaluate the bioactivities of peptides in both *in vitro* and *in vivo* environments, it is imperative to isolate and purify them. However, a common issue that arises after protein hydrolysis is the insufficient yield of the desired peptides. To overcome this challenge, additional processing steps are typically employed to selectively choose and purify the target peptides, ensuring the acquisition of high-quality peptides. The selection method utilized post-hydrolysis is based on the physicochemical and structural characteristics of the constituent peptides, aiming to enhance their bioactivities (Udenigwe and Aluko, 2012). Various approaches can be employed to separate bioactive peptides from a mixture of protein hydrolysates; thus, chromatography and membrane-based separation techniques are commonly used.

Chromatographic techniques, including size-exclusion chromatography, high-performance liquid chromatography (HPLC), reversed-phase HPLC, ion-exchange chromatography, gel electrophoresis, isoelectric focusing, and low-pressure hydrophobic interaction chromatography, can be used for peptide purification by considering the size differences between peptides and other molecules (Samtiya et al., 2021; Udenigwe and Aluko, 2012). Capillary electrophoresis, fast protein liquid chromatography (FPLC), and reverse-phase high-performance liquid chromatography (RP-HPLC) are commonly employed for peptide separation based on their hydrophobic properties (Alashi et al., 2013; Samtiya et al., 2021; Girgih et al., 2013). Moreover, chromatography with selective ion-

exchange columns can acquire peptide fractions with net charges. This processing mean is valuable and beneficial when the disease targets are inactivated by molecules with strong net positive and negative charges. It has been successful in purifying ACE-inhibitory and copper-chelating peptides from plant protein hydrolysates (Udenigwe et al., 2009). The selection of the appropriate separation method depends on the specific properties of the peptides, and a combination of chromatographic techniques, including HPLC and affinity chromatography, is often employed for successful purification.

Membrane-based separation methods utilize porous membranes and applied pressure to separate components in a mixture. These methods include microfiltration, ultrafiltration, nanofiltration, and reverse osmosis, depending on the membrane properties. Ultrafiltration (UF) and nanofiltration (NF) have emerged as highly effective techniques for purifying peptides based on their molecular weight. Research has indicated that the yield, purification, and quality of products obtained through UF and NF separation are comparable to those achieved through chromatographic processes. Additionally, UF and NF offer the advantages of higher productivity and lower cost, making them favorable alternatives for peptide purification (Marson et al., 2020; Alavi and Ciftci, 2023). The retention mechanism is influenced by peptide size and charge. Different membranes with various pore sizes are used for specific fractionation purposes. Operating pressure is inversely related to membrane pore size, with reverse osmosis requiring the highest pressure and microfiltration employing the lowest pressure. Ultrafiltration membranes are effective in concentrating specific peptide fractions with ACE-inhibitory activity (Alashi et al., 2014). After the process of fractionation, the purity of bioactive peptides is commonly

evaluated using mass spectrometry (MS) techniques. Electrospray ionization mass spectrometry (ESI-MS) is utilized to analyze polar compounds, such as proteins (Udenigwe et al., 2016; Panjaitan et al., 2018). On the other hand, matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) is highly effective in characterizing complex protein mixtures sourced from diverse origins. Additionally, the peptides are subjected to sequencing techniques such as tandem MS (MS/MS) or database search to identify them and ascertain their molecular weight (Agyei et al., 2018; Senadheera et al., 2022). The summary of the production, purification, and characterization of bioactive peptides derived from plant seeds is illustrated in Figure 2.4.

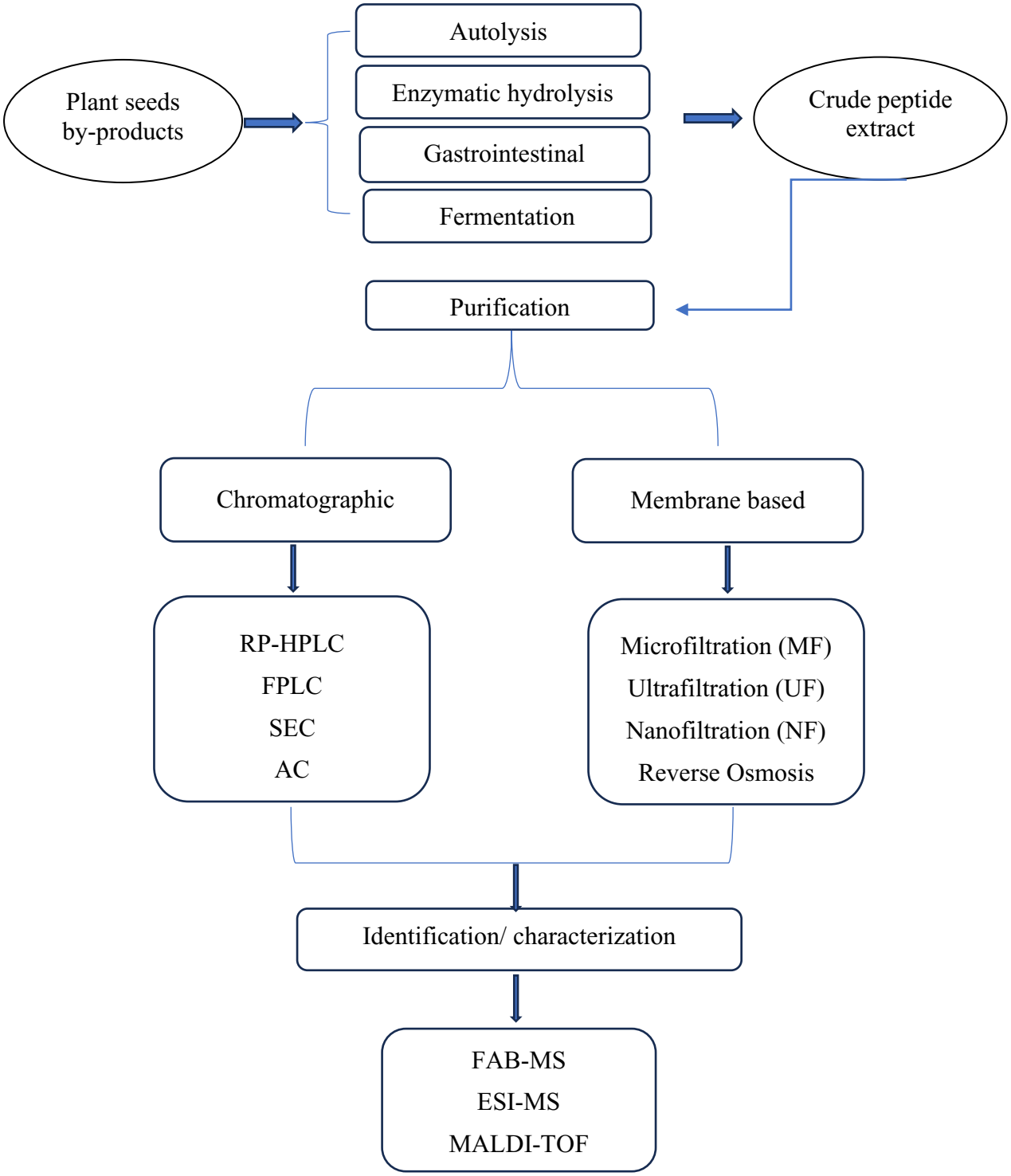

Figure 2.4 The process of producing, purifying, and characterizing bioactive peptides derived from plant seeds adapted from Samtiya et al. (2021); Marson et al. (2020).

RP-HPLC—reversed phase high performance liquid chromatography, FPLC—fast protein liquid chromatography, SEC—size exclusion chromatography, AC—affinity chromatography, CE—capillary electrophoresis, IEC—ion-exchange chromatography, FAB-MS—fast atom bombardment mass spectrometry, ESI-MS—electrospray ionization mass spectrometry, MALDI-TOF—matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

2.6.3 Characteristics of hydrolysates/ bioactive peptides

Following the discovery of the functional properties of bioactive peptides, extensive research has been conducted to explore their beneficial effects on human diseases. Amongst bioactive peptides, one of the most frequently reported bioactivities is antioxidant properties, which follows a range of mechanisms, including chelating metal ions, scavenging free radicals, and exhibiting reducing power (Cumby et al., 2008; Ambigaipalan et al., 2015; Udenigwe and Aluko, 2012). In addition, angiotensin converting enzyme (ACE) and dipeptidyl peptidase IV (DPP IV) inhibitory peptides are well documented as antihypertensive and antidiabetic agents, respectively (Udenigwe and Aluko, 2012; Samtiya et al., 2021; Tavano et al., 2018).

2.6.3.1 Antioxidant activity

The excessive generation of reactive oxygen species and subsequent oxidative stress can contribute to cell damage and the development of diseases such as cancer, diabetes, cardiovascular disease, and obesity (Samtiya et al., 2021). Antioxidants are vital for minimizing oxidative processes in both food systems and in the human body. They play a critical role in slowing down lipid peroxidation and the formation of secondary lipid

peroxidation products, thereby preserving the flavor, texture, and color of stored food products. Additionally, antioxidants help reduce protein oxidation and prevent the interaction between lipid-derived carbonyls and proteins, which can result in changes to protein functionality (Samaranayaka and Li-Chan, 2011; Ambigaipalan and Shahidi, 2015). Plant seeds are rich sources of protein, and thus, potential sources of antioxidant peptides including those of cereals (e.g., rice, wheat), legumes (e.g., soybean, pea), oilseeds (e.g., canola, flaxseed, sesame), and other seeds like quinoa, amaranth, and chia have been explored for antioxidant peptides (Sá et al., 2020; Sá et al. 2021).

Antioxidant peptides are typically oligopeptides, composed of 2-20 amino acid residues. The antioxidative properties of these peptides are primarily associated with their structure, composition, and other relevant factors. Amino acids such as histidine, cysteine, lysine, methionine, tryptophan, and tyrosine play significant roles in facilitating robust antioxidant activity, while aromatic amino acids have the ability to donate electrons to electron-deficient radicals (Udenigwe and Aluko, 2011; Nwachukwu and Aluko, 2019). Moreover, recent research has indicated that low molecular weight peptides generally exhibit superior antioxidant activity compared to larger peptides (Alashi et al., 2014; Hu et al., 2020; Girgih et al., 2011). For example, the smaller-size peptides (<3 kDa) obtained from canola protein isolates demonstrated higher potency as $ABTS^{\cdot+}$ scavengers compared to the larger-sized peptides (>3 kDa) (Alashi et al., 2014). In addition, studies have reported that the DPPH radical scavenging activity of hemp seed peptides was more robust in low molecular weight peptides than in larger peptides (Girgih et al., 2011).

Researchers have highlighted that while there is still a lack of complete understanding regarding the structure-function relationship of antioxidant peptides, it is widely believed that the type and sequence of amino acids play crucial roles in determining the antioxidative properties of peptides (Udenigwe and Aluko, 2012; Zou et al., 2016). For instance, the notable DPPH radical scavenging capability of CSQAPLA, obtained from corn protein hydrolysates, is primarily attributed to the APLA portion of the peptide chain. This is primarily due to the hydrophobic interactions among non-polar amino acid residues, which have been linked to enhanced antioxidant ability (Jin et al., 2016). Peptide hydrophobicity plays a vital role in enhancing radical scavenging activity by facilitating increased interaction with reactive oxygen species (ROS). This was demonstrated by the potent DPPH scavenging ability of WVYY, a peptide derived from hemp seed protein, compared to less hydrophobic peptides like WSY, WYT, SVYT, and PSLAPA (Girgih et al., 2014). This implies that the specific arrangement and composition of amino acids within the peptide sequence are influential factors in determining its ability to exhibit antioxidant activity. Further research is needed to gain a more comprehensive understanding of how different amino acid types and sequences contribute to the antioxidative properties of peptides (Table 2.4).

Table 2.4 Antioxidant bioactive peptides from various plant sources

Plant source	Assay	Peptide sequence	Reference
Rice residue protein	DPPH and ABTS radical scavenging	RPNYTDA, TSQLLSDQ, TRTGDPFF, NFHPQ	Yan et al., 2015
Sweet potato protein	Hydroxyl radical scavenging activity assay	YYIVS, YYDPL, TYQTF, SGQYFL, YMVAIWG	Zhang et al., 2014
Corn gluten meal	DPPH radical scavenging	CSQAPLA, YPKLAPNE, YPQLLPNE	Jin et al., 2016
Chickpea	DPPH radical scavenging, reducing power	TETWNPNHPEL, FVPH	Torres-Fuentes et al., 2015
Hempseed protein	DPPH scavenging and metal chelation activity	WVYY, PSLPA, WSY, WYT, SVYT	Girgih et al., 2014
Pecan meal	DPPH and ABTS radical scavenging, Hydroxyl radical scavenging activity	LAYLQYTDFETR	Hu et al., 2018
Faba bean protein	DPPH and ABTS radical scavenging	TETWNPNHPEL, ALEPDHR, VIPAGYP, PHW, PHY, YVE	Samaei et al., 2020

2.6.3.2 Antihypertensive activity

Hypertension is a globally acknowledged health concern of great significance. The development of hypertension is influenced by various well-established factors, such as obesity, diabetes, and kidney diseases (Udenigwe and Aluko, 2012; Samtiya et al., 2021). Angiotensin-I-converting enzyme (ACE) and renin play a crucial role in the renin-angiotensin-aldosterone system (RAAS), which is responsible for regulating blood pressure and fluid balance in the body (Samtiya et al., 2021). Renin plays a crucial role in converting angiotensinogen to angiotensin I, while ACE is responsible for transforming angiotensin I into angiotensin II, a powerful vasoconstrictor. High levels of angiotensin II contribute to hypertension by increasing blood vessel contraction and reducing relaxation. Thus, inhibiting renin or ACE has become a significant approach for treating hypertension.

Currently, aliskiren is the sole approved renin inhibitor, while several drugs like captopril, enalapril, and lisinopril effectively inhibit ACE and are available for hypertension treatment (He et al., 2013). Nonetheless, the current antihypertensive ACE-inhibitory drugs are associated with adverse side effects such as dry and persistent cough, hyperkalemia, allergic reaction, diarrhea, difficulty in swallowing or breathing, tachycardia, and decrease in the white blood cells. These side effects can potentially impact the patient's overall health, significantly reduce their quality of life, affect life expectancy, and contribute to increased healthcare costs (Malomo et al., 2014; Samtiya et al., 2021; Girgih et al., 2014). There are various reports that plant-based bioactive peptides may be able to replace or complement drugs as antihypertensive agents (Udenigwe and Aluko, 2012; Samtiya et al., 2021; Han et al., 2021). Previous studies have shown that the protein

hydrolysates from various plant seeds such as sunflower protein (Megías et al., 2009), rapeseed protein (He et al., 2016; He et al., 2013), date seed (Ambigaipalan et al., 2015), hemp seed protein (Malomo et al., 2014), quinoa protein (Aluko and Monu, 2003; Mudgil et al., 2020), among others, may be able to do so. Another study demonstrated that bioactive peptides, particularly those with low molecular weight have promising potential in inhibiting ACE, decreasing blood pressure, and preventing hypertension (Gorguc et al., 2020). For instance, the low molecular weight (< 1 kDa) peptide fractions obtained from rapeseed and sesame seeds were the most active against ACE (He et al., 2013; Aondona et al., 2021) .

Studies reported that ACE-inhibitory peptides may share common structural properties, which is the presence of aromatic amino acid residues at the C-terminus and hydrophobic amino acid residues at the N-terminus (Alashi et al., 2013; Acquah et al., 2018). Moreover, a multitude of sequences exhibit ACE inhibitory activity, illustrating the remarkable diversity of peptides that can function as ACE inhibitors. For example, bioactive peptides with antihypertensive activity have been identified in various plant sources, including rice (IHRF), soybean (DLP and DG), and pea (IR, KF, and EF), rapeseed (GHS) (Kontani et al., 2014; Wu, 2002; Li and Aluko, 2010; He et al., 2013), among others (Table 2.5). The efficacy of peptides that show promise as antihypertensive agents in laboratory studies can be compromised when tested in living organisms due to degradation by gastrointestinal enzymes, limiting their absorption and availability, and the complex mixture of peptides in protein hydrolysates makes the identification of specific peptides

challenging. However, derived plant peptides still hold significant potential for developing effective antihypertensive treatments.

Table 2.5 Bioactive peptide with ACE inhibitory activity from various plant sources

Plant source	Peptide sequence	Reference
Rapeseed	LY, TF, RALP	He et al., 2013
Sunflower seed	FVNPQAGS	Megías et al., 2004
Ginko biloba seeds	TNLDWY, RADFY, RVFDGAV	Ma et al., 2018
Soybean	LAIPVNKP, LPHF, SPYP, WL	Kuba et al., 2005
Cañihua seed	LDKDYPKR, RLSAEKGVLYR, LFR	Chirinos et al., 2018
Pea	AKSLSDRFSY	Liao et al., 2019
Hemp seed	WVYY, WYT, SVYT, IPAGV	Girgih et al., 2014
Quinoa bran	RGQVIYVL, ASPKPSSA, QFLLAGR	Zheng et al., 2019

2.6.3.3 Hypoglycemic activity

Diabetes is a significant global metabolic syndrome, and its prevalence has been steadily increasing in recent years. It is classified into two primary types. Type 1 diabetes is an autoimmune condition characterized by the immune system mistakenly attacking and

destroying the insulin-producing beta cells in the pancreas. On the other hand, Type 2 diabetes involves insulin resistance and insufficient insulin production. In Type 2 diabetes, the body becomes less responsive to insulin or fails to produce enough of it to effectively regulate blood sugar levels (Nongonierma and Fitzgerald, 2014; Kehinde and Sharma, 2020). This type is the major form of diabetes, accounting for about 90% of cases diagnosed and is often associated with lifestyle factors such as obesity, physical inactivity, and poor dietary choices. Treatment approaches may include lifestyle adjustments, oral or injectable medications, and, in some cases, insulin therapy (Wang et al., 2019; Zhu et al., 2017; You et al., 2022). Dipeptidyl peptidase IV (DPP-IV) is a well-known enzyme that plays a crucial role in the quick breakdown and splitting of glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). These incretin hormones are closely linked to the synthesis and release of insulin (Han et al., 2021).

Bioactive peptides and protein hydrolysates derived from various plant seeds have demonstrated antidiabetic properties, making them potential candidates for alternative therapies. These peptides exhibit their antidiabetic effects through multiple mechanisms, including the inhibition of dipeptidyl peptidase IV. Enzymatic digestion is employed to isolate antidiabetic hydrolysates and peptides from different plant seeds, which serve as protein sources for this purpose (Kehinde and Sharma, 2020; Samtiya et al., 2021). For example, using Umamizyme G, a protein hydrolysate with DPP-IV inhibitory properties was successfully produced from defatted rice bran. Among the peptides derived from rice bran, LP and IP were specifically identified as the inhibitory peptides responsible for this effect. This indicates that the protein hydrolysate from rice bran has the potential to inhibit

the activity of DPP IV, which is beneficial in the context of managing conditions such as diabetes (Hatanaka et al., 2012). In addition, peptides from soybean (IAVPTGVA, YVVNPDNDEN, YVVNPDNNEN) and lupin proteins (LTFPGSAED, LILPKHSDAD, GQEQSHQDEGVIVR) were capable of inhibiting the activity of DPP IV (Lammi et al., 2016). These peptides are mostly composed of hydrophobic amino acid residues, such as A, G, I, L, and P, which play a crucial role in the inhibitory activity of peptides against DPP IV, contributing to their effectiveness in managing conditions such as diabetes. Moreover, protein hydrolysates derived from quinoa exhibited significant inhibitory activity against DPP IV. Specifically, the hydrolysate fraction with a molecular weight below 1 kDa effectively inhibited DPP IV on Caco-2 cells. Several peptides, including IPI, IPV, VAYPL, and IPIN, competitively inhibited DPP IV. Notably, IPI and IPV demonstrated the highest inhibitory activities against DPP IV. Molecular analysis revealed that these peptides have the potential to form multiple hydrogen bonds, attractive charge interactions, and hydrophobic interactions with the active site residues of DPP IV. This suggests their promising role as DPP IV inhibitors derived from quinoa protein hydrolysates (You et al., 2022). Furthermore, Table 2.6 presents illustrative instances of bioactive peptides obtained from plant seeds, showcasing their potential to lower blood sugar levels (hypoglycemic activity). This emphasizes the promising prospects of harnessing these plant seed-derived compounds to achieve beneficial outcomes in managing blood sugar levels.

Table 2.6 Hypoglycemic activity of bioactive peptides from various plant sources

Plant source	Peptide sequence	Reference
Chlorella vulgaris	VPW, IPR	Zhu et al.,2017
Quinoa protein	IQAEGGLT	Vilcacundo et al., 2017
Porphyra dioica	YLVA	Cermeno et al., 2019
Soy protein	LLPLPVLK, SWLRL, WLRL	Wang et al., 2019
Black beans	AKSPLFATNPLF, FEELN, LSVSVL	Mojica et al., 2018
Wheat gluten proteins	LPF, MPF, MAMG, VAVPV, MAMGL, LAGAP	Ding et al., 2022
Tombul hazelnut	IPI, PGHF, FMRWRDRFL, APGHF, FFFPGPNK	Caglar et al., 2021
Oat globulin	LQAFEPLR, EFLLAGNNK	Wang et al., 2018

2.7 Bioinformatic approach for predicting of potential bioactive peptides

Bioinformatics approaches have revolutionized the discovery and analysis of bioactive peptides by enabling comprehensive and cost-effective investigations. These approaches leverage computational techniques, advanced algorithms, and large-scale peptide datasets to facilitate the discovery, characterization, and understanding of peptides with bioactive properties (Udenigwe, 2014; Agyei et al., 2018). The process starts with the

use of *in silico* tools to mine bioactive peptides, allowing researchers to narrow down their focus to a smaller set of peptide candidates that are expected to possess the desired biological activities with high potency. This approach eliminates the need for guesswork and enables scientists to predict in advance the types of peptides and their potency that can be derived from a given food protein. Additionally, it helps determine the most suitable proteases to be used for releasing these peptides (Carrasco-Castilla et al., 2012; Udenigwe, 2014). In addition to predicting the desirable biological properties and potency of bioactive peptides, it is also crucial to consider the undesirable biological properties, such as allergenicity and toxicity, especially when developing novel bioactive peptide sequences. This knowledge becomes particularly important when the peptides are intended for functional food or pharmaceutical applications (Hayes et al., 2015; Gupta et al., 2015). The process of bioinformatics discovery of bioactive peptides typically begins by obtaining the amino acid sequences of proteins, mainly food proteins, from databases such as UniProtKB, SwissProt, BIOPEP and PepBank (Agyei et al., 2018; Udenigwe, 2014). Once the proteins with known primary sequences are selected, an *in silico* digestion is performed using protein digestion databases that employ protein/peptide cutting functions (Table 2.7). For example, researchers can utilize the BIOPEP database during the *in silico* digestion process to obtain a list of cleaved peptides derived from specific proteins. This information can then be further analyzed and evaluated to identify potential bioactive peptides with desired properties. The database aids in the prediction and selection of bioactive peptides based on their known enzymatic cleavage patterns and activities (Minkiewicz et al., 2019). The BIOPEP database has been extensively utilized for the identification and analysis of bioactive peptides with antioxidant activities antihypertensive activity and DPP IV-

inhibitory activities derived from various sources such as giant grouper roe proteins (Panjaitan et al., 2018), invasive sea grass *Halophila stipulacea* (Kandemir-Cavas et al., 2019), oil seeds protein (Han et al., 2019), flax seeds protein (Langyan et al., 2021), and sea cucumber (Senadheera et al., 2022), among other. In addition, an *in silico* study has shown that oilseed proteins (soybean, flaxseed, rapeseed, sunflower and sesame) have the potential to serve as promising precursors to produce peptides with ACE (angiotensin-converting enzyme) and DPP IV (dipeptidyl peptidase IV) inhibitory activities. Additionally, this study has also revealed the presence of unexplored peptides within oilseed proteins that may play roles beyond ACE and DPP IV inhibition (Han et al., 2019). The results of this study provide valuable insights into the potential applications of oilseed proteins in the development of functional foods, nutraceuticals, or pharmaceuticals targeting ACE and DPP IV inhibition. Further exploration and experimental validation are warranted to uncover and harness the bioactive potential of these peptides for various health-related applications.

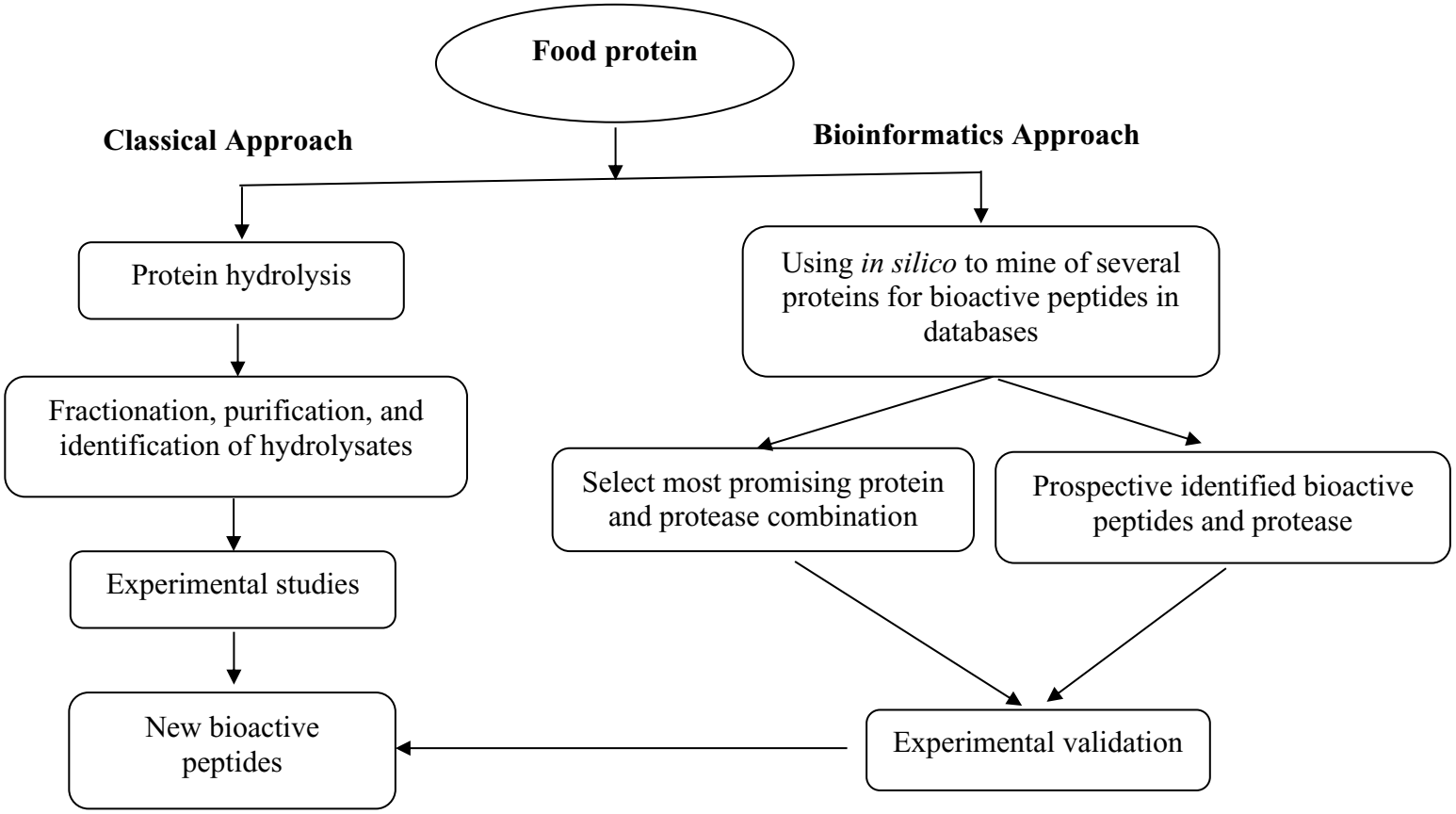

Figure 2.5 Classical and bioinformatics approaches towards the development of new bioactive peptides from food protein adapted from Udenigwe (2014).

Table 2.7 *In silico* approaches to the discovery and analysis of food-derived bioactive peptides adapted from Agyei et al. (2018)

***In silico* approaches**	**Database and tools**
Databases of protein sequences	RCSB Protein Data Bank, https://www.rcsb.org/pdb/home/home.do
	UniProtKB, http://www.uniprot.org/
	NCBI Protein, http://www.ncbi.nlm.nih.gov/protein
	BIOPEP, http://www.uwm.edu.pl/biochemia/
	PepBank, http://pepbank.mgh.harvard.edu/
	BioPD, http://biopd.bjmu.edu.cn/
	SwePep, http://www.swepep.org/
	EROP-Moscow, http://erop.inbi.ras.ru/
	MilkAMP, http://milkampdb.org/
	PeptideDB, http://www.peptides.be/
	AMPer, http://marray.cmdr.ubc.ca/cgi-bin/amp.pl
Databases of proteolytic enzymes and *in silico* digestion platforms	BIOPEP, http://www.uwm.edu.pl/biochemia/index.php/en/biopep
	PeptideCutter, http://web.expasy.org/peptide_cutter/
	POPS, http://pops.csse.monash.edu.au/pops-cgi/index.php
	Enzyme Predictor, http://bioware.ucd.ie/~enzpred/Enzpred.php
	PeptideRanker, http://bioware.ucd.ie/~compass/biowareweb/
Prediction of potential bioactivity	BIOPEP, http://www.uwm.edu.pl/biochemia/index.php/en/biopep
	AntiBP2, http://www.imtech.res.in/raghava/antibp2/
	PeptideLocator, http://bioware.ucd.ie/

Toxicity/allergenicity prediction	ToxinPred, http://www.imtech.res.in/raghava/toxinpred/ AlgPred, http://www.imtech.res.in/raghava/algpred/ Allerdictor, http://allerdictor.vbi.vt.edu/ EPIMHC, http://bio.dfci.harvard.edu/epimhc/ SORTALLER, http://sortaller.gzhmu.edu.cn/ ProPepper, https://propepper.net/

2.8 Applications of protein isolates and hydrolysates/bioactive peptides in food

As the global population grows, there is a growing need to rely on plant proteins when animal-derived proteins are insufficient to meet the demand. The food and processing industries place great importance on understanding the physicochemical properties, structural characteristics, amino acid composition, and functional attributes of plant-based proteins. This understanding is essential for effectively utilizing plant proteins as a vital resource in meeting the nutritional needs of a growing population (Kumar et al., 2022). Hence, protein plant are highly purified protein forms that contain a higher protein content and improved digestibility. They have become a prominent and cost-effective source of protein, particularly for athletes, bodybuilders, vegetarians, and individuals seeking high-quality proteins. They are widely used in beverages and dairy industries, and infant foods as functional ingredients. They can be incorporated into products such as protein bars, protein shakes, sports drinks, infant formulas, vegetarian/vegan food products, and meat substitutes. Protein isolates provide nutritional value, improve texture, enhance flavor, and contribute to the protein content of these food and beverage products (Filho and Egea, 2021; Lena et al., 2023; Loveday, 2019). Numerous plant sources have been extensively investigated as potential protein supplements. These include a wide range of options such as legumes (chickpea, cowpea, soybean, pea, lupin), cereals (wheat, rice, sorghum, minor millets, maize, barley), pseudocereals (amaranth, buckwheat, and quinoa), seeds (sunflower, pumpkin, sesame, flaxseed), and among others (Sá et al., 2020). For examples, quinoa protein has been used in cupcakes improving in firmness and water activity (Lopez-Alarcon et al., 2019). Oilseed meals and cakes such as rapeseed, sunflower, camelina,

which are obtained as co-products during the extraction of seed oil, are particularly intriguing due to their notable protein content (Filho and Egea, 2021; Lena et al., 2023; Tavarini et al., 2021). For instance, the addition of rapeseed protein isolates in chicken breast and bread led to sensory properties that remained unchanged or even improved, along with higher nutrient density, especially in terms of proteins and minerals (Lena et al., 2023). These co-products have garnered attention because they offer an opportunity for their utilization in both animal and human nutrition, thereby contributing to the sustainable utilization of oilseeds.

Furthermore, Hydrolysates and bioactive peptides derived from plant seeds mentioned possess a wide range of potential applications as functional food ingredients, nutraceuticals, and pharmaceuticals, contributing to human health improvement and disease prevention. These peptides exhibit diverse activities that have been reported, including anti-hypertensive effects, acting as opioid agonists or antagonists, immunomodulatory properties, antithrombotic actions, antioxidant capabilities, anti-cancer properties, antimicrobial activities, and enhancing nutrient utilization (Ambigaipalan and Shahidi, 2015; Cumby et al., 2008; Karami and Akbari-Adergani, 2019; Kumar et al., 2022). For example, the addition of date seed flour hydrolysate resulted in a significant increase in the moisture content and notable improvement in the texture of the muffins (Ambigaipalan and Shahidi, 2015). In addition, faba protein hydrolysates have been incorporated into orange juice and apple juice as a valuable source of amino acids and peptides that possess antioxidant properties. This addition enhances the nutritional value and antioxidant capacity of the juices, providing consumers with a healthier and more

functional beverage option (Samaei et al., 2020). In summary, before using protein isolates and hydrolysates as functional food additives, it is essential to conduct thorough investigations into their adaptability with various food matrices, gastrointestinal stability, bioavailability, and long-term stability. These factors are critical for ensuring the efficacy and safety of protein ingredients in functional food applications. Further research and studies are needed to comprehensively assess these aspects and determine the suitability of protein isolates and hydrolysates as functional ingredients in food formulation and nutraceutical.

2.9 Conclusion

The summary highlights that protein isolates improve properties such as water holding capacity, foaming capacity, emulsifying properties, and solubility in food products. Moreover, the potential of food proteins-derived bioactive peptides as modulators in the body, with various functional activities such as antioxidant, ACE inhibitory, and hypocholesterolemia activities are clearly demonstrated. Protein hydrolysates' functionality and biological activities depend on the peptide structure and amino acid sequence. Maximizing functionality requires identifying significant variables in the hydrolysis process. Additional research is needed to ensure the safety and optimum quality of foods containing bioactive peptides, to develop techniques for large-scale production, and to stabilize peptide structures and retain their biological activities in different food matrices. By-products from oilseed extraction, particularly those from camelina, and sophia, show potential as a source of high-quality functional food ingredients. Furthermore, their techno-functional and sensory attributes can be modified using physical methods, thermal

treatments, fermentation, and enzymatic processes, allowing for diverse applications in plant-based food products. Enzymatic protein hydrolysates are vital as they offer better control and fewer secondary reactions. Different hydrolysates with diverse functionalities can be developed for production of foods by optimizing variables like enzyme specificity and hydrolysis conditions. Limited literature exists on camelina and sophia seed meals that focuses on protein isolates and their hydrolysates, with functional properties and antioxidant, ACE-inhibitory, and DPP IV-inhibitory activities. More research is needed to identify and characterize bioactive peptides from camelina and sophia meal protein hydrolysates with antioxidant, ACE-inhibitory activities, DPP IV-inhibitory activities, antimicrobial, and anticancer properties. Such research would enhance the value of camelina and sophia meal proteins in food manufacturing.

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CHAPTER 3

Functional properties of protein isolates from camelina (*Camelina sativa* (L.) Crantz) and flixweed (sophia, *Descurainis sophia* L.) seed meals

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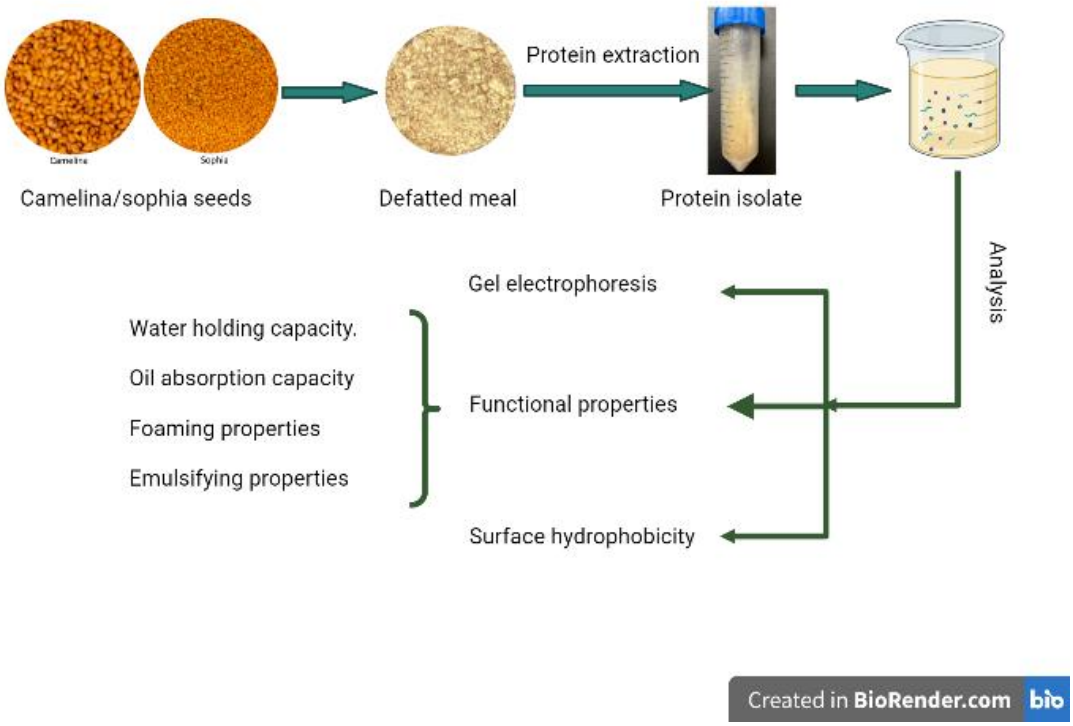
3.1 Abstract

Camelina and flixweed (sophia) seed protein isolates were prepared using both the conventional extraction and ultrasonic-assisted extraction methods at 40 kHz for 20 min, and their functional properties investigated. SDS-PAGE showed that both ultrasound-assisted and conventional extractions resulted in a similar protein profile of the extract. The application of ultrasound significantly improved protein extraction/content and functional properties (water holding capacity, oil absorption capacity, emulsifying foaming properties, and protein solubility) of camelina protein isolate and sophia protein isolate. The water-holding and oil absorption capacities of sophia protein isolate were markedly higher than those of camelina protein isolate. These results suggest that camelina protein isolate and sophia protein isolate may serve as natural functional ingredients in the food industry.

Keywords: Camelina seed, sophia seed, protein isolate, functional properties, ultrasound treatment

Graphic abstract



3.2 Introduction

Proteins play a crucial role in both biological systems and human nutrition. Compared with animal-based proteins, plant-derived proteins are currently receiving much interest as they are sustainable and readily available with much economic attraction (Deng et al., 2019; Du et al., 2018). Hence, diversified sources of plant proteins increasingly address the worldwide requirement as an ingredient in the food industry.

Over the last few years, attraction to proteins from food processing by-products has intensified. Protein isolates play an integral part in the development of food with desirable functional properties (Yagoub et al., 2017). Many efforts have been made to develop efficient methods to produce a high-quality protein with good utilization value. Camelina

(*Camelina sativa* (L.) Crantz) and flixweed, also known as sophia (*Descurainis sophia* L.) belong to the family Brassicaceae and may serve as novel sources of plant protein. In addition, camelina is a new potential oilseed source in North America, particularly for use in aquaculture feed. Camelina meal is the by-product of the oil extraction process from camelina seeds. Defatted camelina meal contains approximately 45% protein, residual crude fat 4.9%, up to 15% insoluble fiber, up to 10% carbohydrates, 3% minerals, and approximately 4% phytochemicals, dominated by phenolics and other compounds such as vitamins (Das et al., 2014; Rahman et al., 2018). Furthermore, the nutritional quality of camelina protein is similar to that of canola protein and competes with soy protein for some applications targeting the use of plant proteins (Li et al., 2015). However, there are very few studies on extracted proteins and hydrolysates from camelina (Boyle et al., 2018; Li et al., 2014).

Sophia seeds have been used as a traditional medicine to relieve cough and chest discomfort, prevent asthma, and treat cancer. It can be found throughout Canada as well as in Iran and China (Hadinezhad et al., 2015). The seed contains 28% protein, 33% oil, and 4% ash (Rahman et al., 2018). Their amino acids include aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, and arginine (Mohamed and Mahrous, 2009). Some studies have investigated the fatty acid profile and phenolic compounds of sophia seeds (Rahman et al., 2018). However, no research has been carried out to determine proteins in sophia seeds, and to evaluate their functional properties.

For isolating and recovering proteins, alkaline extraction has become the most popular technique for the preparation of proteins because of its simplicity and cost-effectiveness (Pastuszewska et al., 2000; Phongthai et al., 2016). However, this technique has some potential problems, such as the presence of complex protein constituents with diverse isoelectric points and a wide range of molecular weight distribution (Berot et al., 2005). As a result, an efficient extraction technique plays an essential role in extracting proteins. Numerous studies have shown that ultrasonic-assisted extraction (UAE) is one of the most successful extraction methods because it provides some potential benefits such as short extraction time, high extraction yield, low level of solvent use, and the improvement in solubility (Yagoub et al., 2017; Zou et al., 2017). Recent studies have also reported the effects of ultrasound on structural and functional properties of sunflower protein isolates (Malik and Saini, 2018; Malik et al., 2017), walnuts (Zhu et al., 2018), faba beans (Martinez-Velasco et al., 2018), and canola protein isolates (Flores-Jimenez et al., 2019). Additionally, the sonicated protein extraction increased gelation capacity while decreasing foaming capacity (Ly et al., 2018). This could lead to improved functional and nutritional properties of food proteins developed by using ultrasonic-assisted technology. However, there are no studies on the effects of ultrasound treatment on the quality of camelina protein isolate (CPI) and sophia protein isolate (SPI). Therefore, more research is needed to fill the existing gap about the functional properties of camelina and sophia protein isolates in order to expand their potential use by the food industry. In this study, CPI and SPI were produced by an ultrasonic-assisted extraction method and the results are compared with the commonly used alkaline extraction procedure for comparative purposes.

3.3 Materials and methods

3.3.1 Materials

Camelina and flixweed, sophia, seeds were used in this study. Camelina seeds were obtained through Professor Parrish, Memorial University from Linnaeus plant sciences INC, Saskatoon, SK, Canada. Sophia seeds were from Daghdagh Abad, near Hamedan city in Iran, and were purchased from the Tavazo store in Toronto, ON, Canada. All chemicals used were obtained from Fisher Scientific Ltd. (Ottawa, ON, Canada) or Sigma-Aldrich Canada Ltd (Oakville, ON, Canada).

3.3.2 Defatting

Camelina/ sophia seed samples were defatted with hexane at a 1:5 (w/v) ratio for 5 min in a Waring blender at room temperature. The mixture was filtered through Whatman No. 1 filter paper with a Buchner funnel assisted by vacuum filtration. The above procedure was repeated three times, and samples were then air-dried and stored at -20^{o}C before protein isolation.

3.3.3 Preparation of protein isolate

3.3.3.1 Protein extraction from defatted samples by pH solubilization

Protein isolates were extracted from defatted meals according to Chavan et al. (2001) with some modification. The defatted samples were suspended in distilled water (DW) (3.0%, w/v). The mixture was stirred with a magnetic stirrer for 30 min and the pH was then adjusted to 12 by the addition of a known amount of 2 M NaOH and stirring for another 60 min at room temperature. Under similar conditions, the residues were re-

extracted two more times with distilled water (DW). The supernatants were combined, and the pH was adjusted to 4.5 with the addition of 2 M HCl and then centrifuged at 10,000 x g for 30 min at 4^{o}C to precipitate the protein. The pellets were collected and then washed twice with DW. The precipitated protein was redispersed in DW, and the pH was adjusted to 7.0 with 1 M NaOH. The extracted proteins were freeze-dried and stored at -20^{o}C for subsequent analyses.

3.3.3.2 Ultrasonic-assisted extraction of protein from defatted samples

For the ultrasound treatment, the defatted samples were mixed in DW, and the pH of the solution was adjusted to 12 using 2 M NaOH. In the next steps, the suspended sample was placed in an ultrasound bath (180 W, 40 kHz, 20 min) to extract the proteins. After extraction, the procedure followed was the same as the one under section 3.3.3.1. The protein content in the supernatant was determined according to the Bradford (1976) method.

3.3.4 Gel electrophoresis

The protein profile of CPI/SPI was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), according to the method of Laemmli (1970) with 5% stacking gel and 12% separating gel. Samples of CPI and SPI were each mixed with buffer (Tris-HCl, pH 8.8). Twenty microliters of the prepared solutions were added to 1 mL buffer (distilled water, 0.5 M Tri–HCl pH 6.8, glycerol, 10% SDS, 1% bromophenol blue, and beta-mercaptoethanol) and incubated in boiling water for 5 min and then centrifuged at 12,000 x g for 30 s. Ten microliters of samples were applied to the sample wells. The standard protein marker contained (250, 130, 100, 70, 55, 35, 25, 15,

and 10 kDa) and was used as a molecular weight standard. Electrophoretic migration was monitored at a constant current (100V-200V) for 1.5–2 h. The gel was stained with Coomassie Brilliant Blue R-250 for 60 min. The stained gel was destained by frequently changing the fixing solution until the excess stain disappeared.

3.3.5 Surface hydrophobicity

Surface hydrophobicity was determined according to the bromophenol blue (BPB) binding method reported by Tontul et al. (2018). BPB has been shown to bind to the same hydrophobic sites on proteins as polarity-sensitive fluorescence probes. Freeze-dried CPI/SPI (5 mg/mL, w/v) was dispersed in 20 mM pH 7 phosphate buffer. To the protein solution (1 mL) was added 200 µL BPB solution (1 mg/mL BPB in distilled water). The control consisted of a 200 µL BPB solution and 1mL phosphate buffer. Test and control samples were agitated for 10 min and then centrifuged at 2,000 x g for 15 min. The absorbance of the supernatants was read at 595 nm using a spectrophotometer. Surface hydrophobicity was determined from the bound BPB using the following formula.

$$\text{Surface hydrophobicity } (\mu g) = 200\ \mu g \times (A_{control} - A_{sample})/A_{control}$$

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

3.3.6 Functional properties

3.3.6.1 Protein solubility

Protein solubility was measured according to the method described by Ambigaipalan and Shahidi (2015) with some modifications. Freeze-dried CPI/SPI (1 mg/mL, w/v) was dispersed in distilled water, and its pH was adjusted to 2, 3, 4, 5, 6,7, 9,

and 12 by the addition of (1M or 6M) HCl or NaOH. The mixture was stirred for 60 min and then centrifuged at 7,500 x g for 15 min. The protein contents of supernatants were determined using the Bradford (1976) method with bovine serum albumin as a standard, and the total protein content in the sample was determined by solubilizing the sample in 0.5 M NaOH. The solubility of CPI/SPI was expressed as the percentage ratio of the protein content of supernatant to the total protein content using the following equation:

$$\text{Solubility (\%)} = \frac{\text{(Protein content in supernatant)} \times 100}{\text{Total protein content in sample}}$$

3.3.6.2 Water holding capacity (WHC)

The determination of water holding capacity was carried out according to the method of Deng et al. (2019) with slight modifications. The CPI/SPI (0.2 g) was mixed with 5 mL of distilled water in a pre-weighed centrifuge tube. The mixture was vortexed for 2 min and then allowed to stand for 60 min at room temperature. The supernatant was carefully discarded after the dispersion was centrifuged at 8,000 x g for 20 min. The total weight of the precipitate and the centrifuge tube was measured. The WHC was calculated according to the following equation:

$$\text{WHC (g/g)} = (W_2 - W_1)/W_0$$

Where:

W_0 = The weight of protein isolate.

W_1 = The weight of centrifuge tube and protein isolate sample.

W_2 = The weight of the centrifuge tube and precipitated protein isolate after absorbing water.

3.3.6.3 Oil absorption capacity (OAC)

For the determination of the oil absorption capacity of CPI/SPI, the method of Deng et al. (2019) was followed. CPI/SPI (0.2 g) was added to 5 mL commercial corn oil in a pre-weighted centrifuge tube and then mixed for 2 min and allowed to stand for 60 min at room temperature. The mixture was centrifuged at 8,000 x g for 20 min, and the supernatant was discarded. The OAC was calculated according to the following equation:

$OAC\ (g/g) = (W_2-W_1)/W_0$

Where:

W_0 = The weight of protein isolate.

W_1 = The weight of centrifuge tube and protein isolate sample.

W_2 = The weight of the centrifuge tube and precipitated protein isolate after absorbing oil.

3.3.6.4 Foaming properties

The method described by Shahidi et al. (1995) and Elsohaimy et al. (2015) was used with some modification. CPI/SPI (1 g) was dispersed in distilled water, and the mixture was then adjusted to different pH values (2, 4, 6, 8, 10). The dispersion was homogenized at 16,000 rpm for 2 min. The volume of the foam was recorded at 0 min and 10 min, respectively. Foam capacity (FC) was expressed as the percentage volume increase after homogenization, and foam stability (FS) was determined as the volume of foam remaining after 10 min quiescent periods according to the following equation:

$FC = (V_0\text{-}V) \times 100/V$

$FS = ((V_1\text{-}V) \times 100)/ (V_0\text{-}V)$

Where: V(mL), V_0 (mL), V_1(mL) are the volume of foam at initial, 0 min, and 10 min, respectively.

3.3.6.5 Emulsifying properties

The emulsifying activity index (EAI) was determined according to Hang et al. (2014) and Tontul et al. (2018) with slight modification. Briefly, CPI/SPI was dispersed in distilled water (0.5 % w/v) and then adjusted to pH 7 using NaOH or HCl (0.1 or 1 M). Fifteen milliliters of the dispersion were then added to 5 mL of corn oil and the mixture was blended using a high-speed homogenizer at 16,000 rpm for 2 min. After homogenization, 25 μL of the emulsion were taken immediately and transferred to a tube, and diluted with 5 mL SDS (0.1%). The absorbance of the diluted emulsion was read at 500 nm using a spectrophotometer. The emulsion stability index (ESI) at 10 min was measured using the same procedure described. EAI and ESI were calculated using the following equation:

$EAI\ (m^2/g) = (2 \times 2{,}303 \times A_0 \times DF)/ (C \times \varphi \times 10000)$

$ESI\ (min) = A_0 x10/(A_0\text{-}A_{10})$

where A_0, A_{10} are the absorbance of the emulsion 0 min, 10 min, respectively after emulsification, DF was the dilution factor, C was the concentration of the sample (g/mL), φ was the volume fraction of the oil in the emulsion.

3.3.7 Statistical analysis

All experiments were conducted in triplicates and data were reported as mean ± standard deviation. One-way ANOVA was performed, and means were compared by using Tukey's HSD test ($p < 0.05$), SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

3.4 Results and discussion

3.4.1 SDS-PAGE profile of protein extract

Two types of seed storage proteins are abundant in Brassicaceae oilseeds: legumin-type globulins (12 S or cruciferin) and napin-type albumins (2S or napin). They account for 60 and 20% of the total proteins in mature seeds, respectively (Wanasundara, 2011). Figure 3.1 indicates the electrophoretic protein profiles obtained from the meal of camelina and sophia meal. In camelina and sophia protein isolates (AE & UAE), there were two major bands: 15-25 kDa and 25-35 kDa. These bands refer to napin and cruciferin, respectively (Tan et al., 2011; Wanasundara, 2011). The profile of camelina protein isolates is similar to those observed by Boyle et al. (Boyle et al., 2018).

The protein band patterns for alkaline extraction (AE) and ultrasonic-assisted extraction (UAE) proteins were similar. Therefore, these gels showed that the primary structure of the protein in CPI and SPI has not been altered by ultrasonic-assisted extraction. similar results were recently observed in the ultrasonic-assisted extraction of proteins from chickpea, kidney bean, and soybean (Byanju et al., 2020).

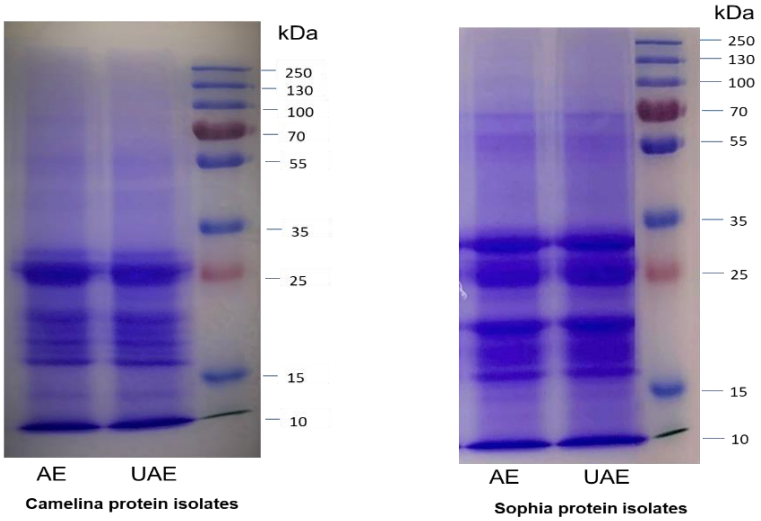


Figure 3.1 SDS-PAGE profile of protein extract of camelina and sophia prepared using both the alkaline extraction (AE) and ultrasonic-assisted extraction (UAE)

3.4.2 Surface hydrophobicity

Surface hydrophobicity (SH) is an important characteristic that influences protein functionality. Higher surface hydrophobicity means that protein isolates have stronger surfactant properties (Mune and Sogi, 2015; Tontul et al., 2018). It was found that the extract methods had different effects on the hydrophobicity surface of protein isolates from camelina and sophia seed meal. The SH of both camelina and sophia protein isolates prepared by UAE was significantly higher than that prepared by AE ($p < 0.05$). The highest SH for sophia protein isolates prepared by UAE was observed (98.16 µg BPB), and the lowest for soy protein isolates as a reference (84.38 µg BPB) (Table 3.1). Significant differences in SH existed among extracted protein from different sources as reported (Mune and Sogi, 2015; Tontul et al., 2018). Therefore, the variations in surface hydrophobicity of extracted protein could be due to changes in protein structure caused by ultrasound. It was

reported the molecular structure of the protein is unfolded under ultrasound, thereby revealing the hydrophobic groups (Wang et al., 2020).

Table 3.1 Surface hydrophobicity (μg bound BPB) of camelina protein isolates (CPI) and sophia protein isolate (SPI)

Sample	BPB bound (μg)
CPI (AE)	90.39 ± 0.40c
CPI (UAE)	93.71 ± 0.10b
SPI (AE)	93.15 ± 0.40b
SPI (UAE)	98.16 ± 0.45a
Soy PI	84.38 ± 0.65d

Values are presented as the mean ± SD of each treatment in triplicate

In the same column, means not connected by the same letter are significantly different at $p < 0.05$

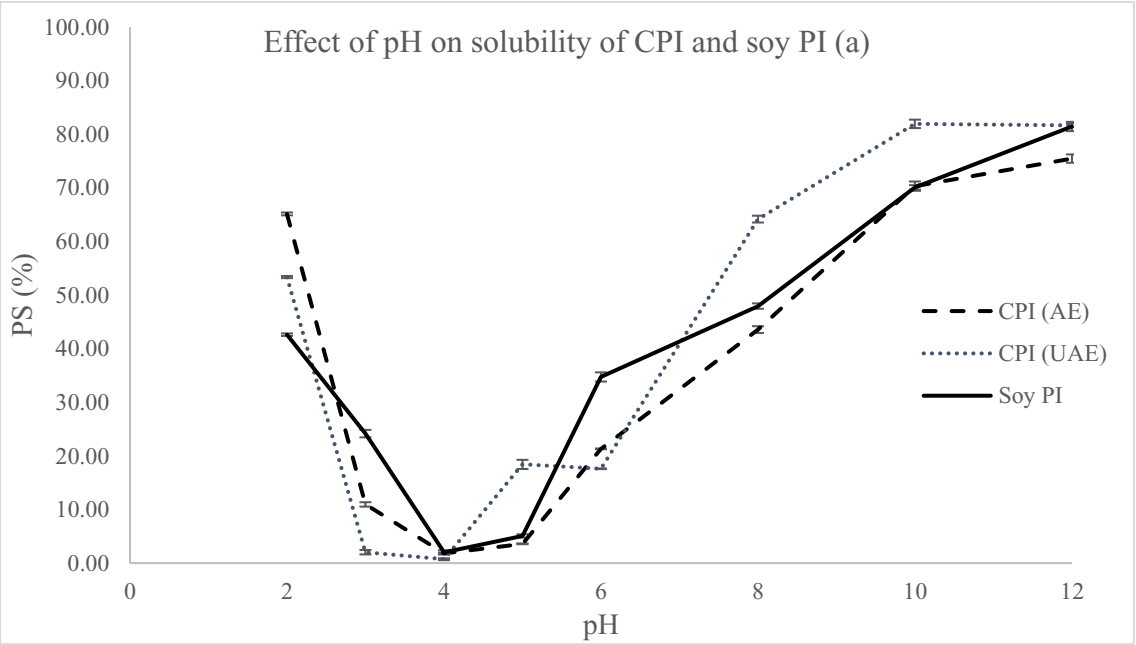
3.4.3 Functional properties

3.4.3.1 Protein solubility

Solubility is one of the most critical characteristics of protein isolates in food formulations as it relates to other functional properties, especially in foams, emulsions, and gels. It also influences the color, texture, and sensory quality of products (Deng et al., 2019; Tontul et al., 2018). According to Figure 3.2, the protein solubility of CPI and SPI shows a typical U-shaped curve within the controlled pH range of 2–12. The PS of the samples were significantly affected by pH and indicated a minimum solubility near pH 3-5, the isoelectric point region for these products. The low PS near the isoelectric point is due to

the balance of positive and negative charges which reduces the electrostatic repulsion among the protein molecules and leads to aggregation and precipitation. In addition, the PS of samples increased gradually with the increase in pH (5–12), and CPI was most soluble at pH 12. These results also correspond to previous studies on different sample types such as rapeseed protein (Dong et al., 2011), quinoa protein isolate (Elsohaimy et al., 2015), mung bean protein (Du et al., 2018), chickpea protein isolate (Tontul et al., 2018), and Chinese quince seed protein isolate (Deng et al., 2019).

Figure 3.2 (a&b) also shows that the PS of soy PI was higher than both CPI (AE) and SPI (AE), however, it was lower than that of UAE. Thus, the solubility of UAE protein isolates improved significantly compared with AE at the pH range of 6-12. Many factors, including the molecular size and composition of protein, influence protein solubility. The decreased particle size caused by ultrasound is likely to increase protein solubility due to better interaction between protein and water (Wang et al., 2020; Yu et al., 2019; Zhang et al., 2018).



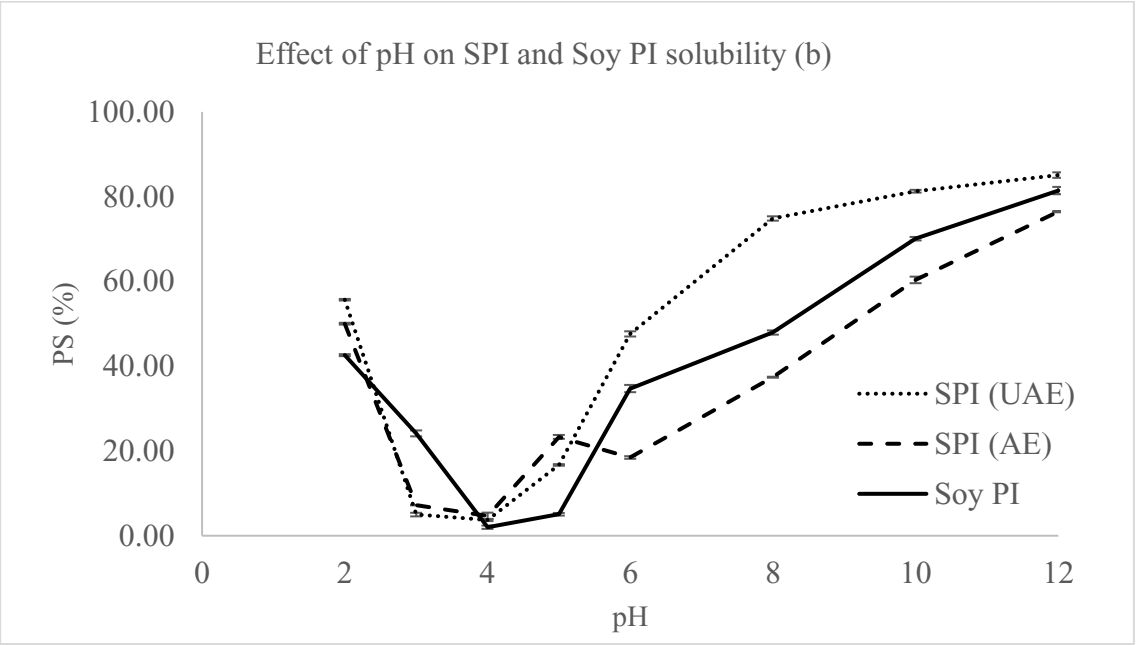


Figure 3.2 (a &b) Protein solubility (PS) of camelina and sophia obtained by alkali extraction (AE) and ultrasound-assisted alkali extraction (UAE) and soy protein isolate (Soy PI) as affected by pH.

Data presented are means of triplicate analyses ± standard deviation ($p<0.05$)

3.4.3.2 Water holding capacity and oil absorption capacity

Water holding capacity is a measure of the ability of proteins to associate gravity with water and it is closely associated with food products' texture, mouthfeel, and viscosity (Deng et al., 2019). The WHC of CPI and SPI is significantly higher than that of soy PI (Table 3.2) ($p<0.05$). Besides, the WHC of CPI (UAE) increased by 20.3% compared with that of CPI (AE) while the WHC of SPI prepared UAE was significantly unchanged. This can be explained by the decrease of the particle size and the improvement of protein solubility for samples extracted by UAE (Wang et al., 2020). Therefore, CPI and SPI could be used to possibly replace soy protein isolates in certain foods.

In general, the oil absorption capacity of proteins represents their ability to bind to oil. It is closely correlated with flavor retention, shelf life, and emulsifying properties. This characteristic may be influenced by factors such as amino acid composition, the type of oil employed, and hydrophobicity, as well as the method used to extract the protein (Deng et al., 2019; Dong et al., 2011). The results in Table 3.2 show that the oil adsorption ability of CPI and SPI was better than that of soy PI. Compared with AE, the OAC of CPI (UAE) and SPI (UAE) were more enhanced by 29.52% and 16.95%, respectively. The improved OAC could be due to the exposure of hydrophobic groups after using UAE. The surface hydrophobicity of CPI and SPI using UAE increased in this analysis, which may make up for the increased OAC of protein isolates.

Table 3.2 Water holding capacity (WHC) and oil absorption capacity (OAC) of camelina protein isolate (CPI), sophia protein isolate (SPI) prepared by alkaline extraction (AE), and ultrasound-assisted alkali extraction (UAE) and soy protein isolate (Soy PI)

Sample	Functional properties	
	WHC (g/g)	OAC (g/g)
CPI (AE)	5.62 ± 0.16c	8.06 ± 0.19d
CPI (UAE)	6.76 ± 0.25a	10.44 ± 0.05b
SPI (AE)	6.32 ± 0.17ab	9.91 ± 0.23c
SPI (UAE)	6.04 ± 0.12bc	11.59 ± 0.27a
Soy PI	4.39 ± 0.16d	5.28 ± 0.12e

Values are presented as the mean ± SD of each treatment in triplicate.

In the same column, means not connected by the same letter are significantly different at $P<0.05$.

3.4.3.3 Foaming properties

Foaming properties are correlated with the ability to decrease surface tension at the water-air interface and are strongly linked to the protein structure. Also, the foam capacity and foam stability are strongly correlated with protein solubility. The higher protein solubility improves water-protein interactions and helps to unfold the protein structure, therefore enhancing air encapsulation (Mundi and Aluko, 2012; Zhang et al., 2018). As shown in Figure 3.3 and Figure 3.4, the lowest FC and FS in all samples was at pH 4 which is the point of least protein solubility. It may be because the ability of proteins to diffuse to form bubbles in the water/air interface is limited. However, it tends to decrease from pH 2

to pH 3 and increase in the pH 5 - 10 range. The results correspond with the reported protein solubility within the pH 2-10 range. With UAE, the FC and FS of SPI were significantly higher than that of AE while they were relatively unchanged in CPI. Ultrasound-assisted alkali extraction could increase foam formation causing changes in protein structure and increase surface hydrophobicity (Wang et al., 2020).

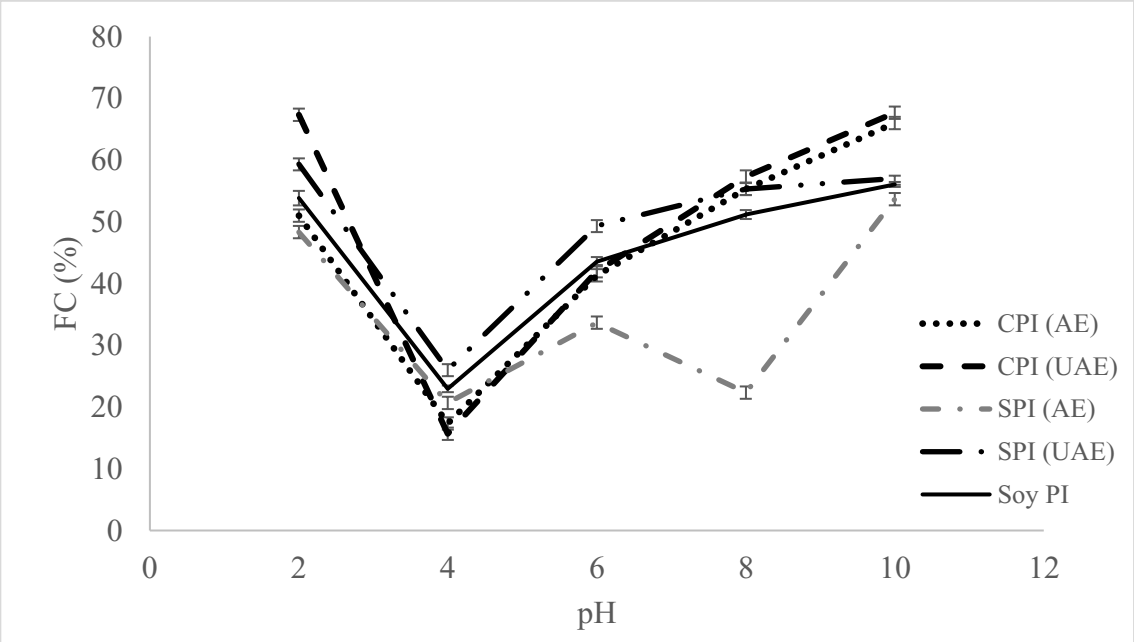


Figure 3.3 Effect of pH on foaming capacity (FC) of camelina protein isolate (CPI) and sophia protein isolate (SPI) prepared by alkaline extraction (AE) and ultrasound-assisted alkali extraction (UAE)

Data presented are means of triplicate analyses ± standard deviation ($p<0.05$)

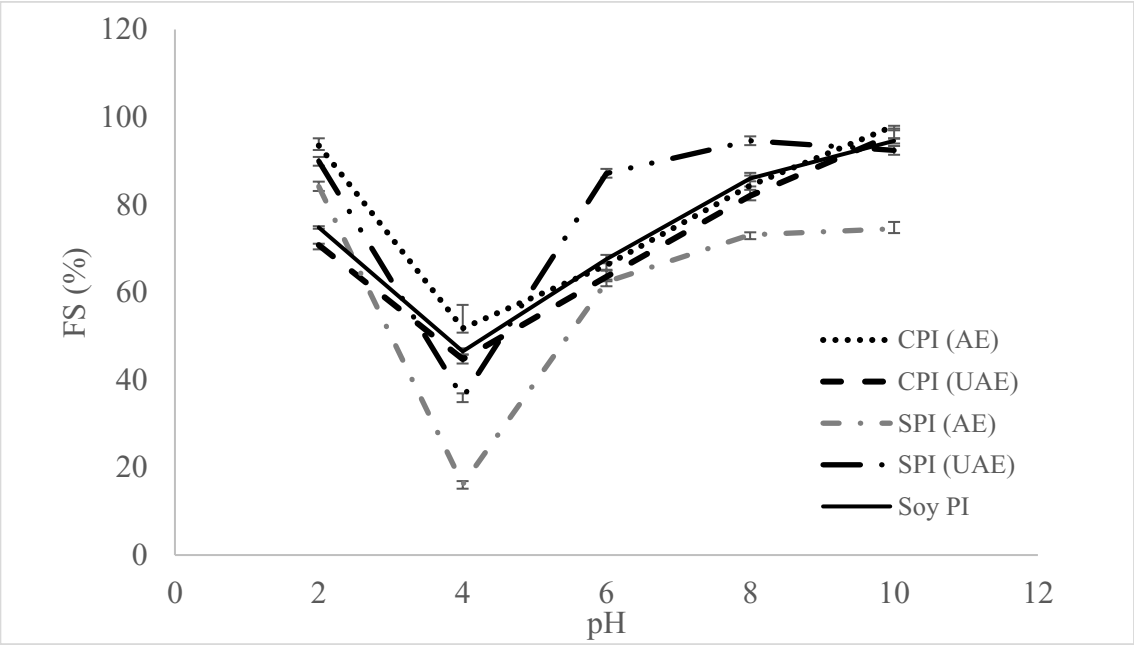


Figure 3.4 Effect of pH on foam stability (FS) of camelina protein isolate (CPI) and sophia protein isolate (SPI) prepared by alkaline extraction (AE) and ultrasound-assisted alkali extraction (UAE)

Data presented are means of triplicate analyses ± standard deviation ($p<0.05$)

3.4.3.4 Emulsifying properties

The emulsifying ability index (EAI) and emulsion stability index (ESI) of CPI at various pH were observed as shown in Figures 3.5 and 3.6, using soybean protein isolate (soy PI) as a reference. The EAI of all samples showed U shape curves within the pH 2- 10 range related to the relationship between protein solubility and pH. Figure 3.5 shows that the lowest EAI was at pH 4, close to the isoelectric point. Besides, the EAI of all samples decreased rapidly when the pH increased from 2 to 4, while there was a sequential increase of EAI within the pH 6-10 range and reached the highest value at pH 10. The results were similar to those reported for canola/rapeseed (Dong et al., 2011; Tan et al., 2011). The EAI

of CPI (UAE) was significantly higher than that of CPI (AE), while the trend of EAI in both SPI (UAE) and SPI (AE) was similar and remained unchanged. The ultrasound-assisted extraction method affects the structure of protein isolates by enhancing molecular flexibility and surface hydrophobicity (Wang et al., 2020). Figure 3.6 shows the lowest of ESI at pH 4, near the isoelectric point, while the highest was at pH 6. The low ESI could be due to the poor solubility, unsatisfactory hydration, and weak electrostatic repulsion among the protein molecules that were insufficiently strong to prevent oil droplet aggregation. Moreover, the EAI of all samples was higher than that of soy PI at most tested pH values, except CPI (AE) for pH 6-10. This suggests the CPI and SPI prepared by UAE emulsifying ability, making it a good candidate emulsifying agent for use in the food industry.

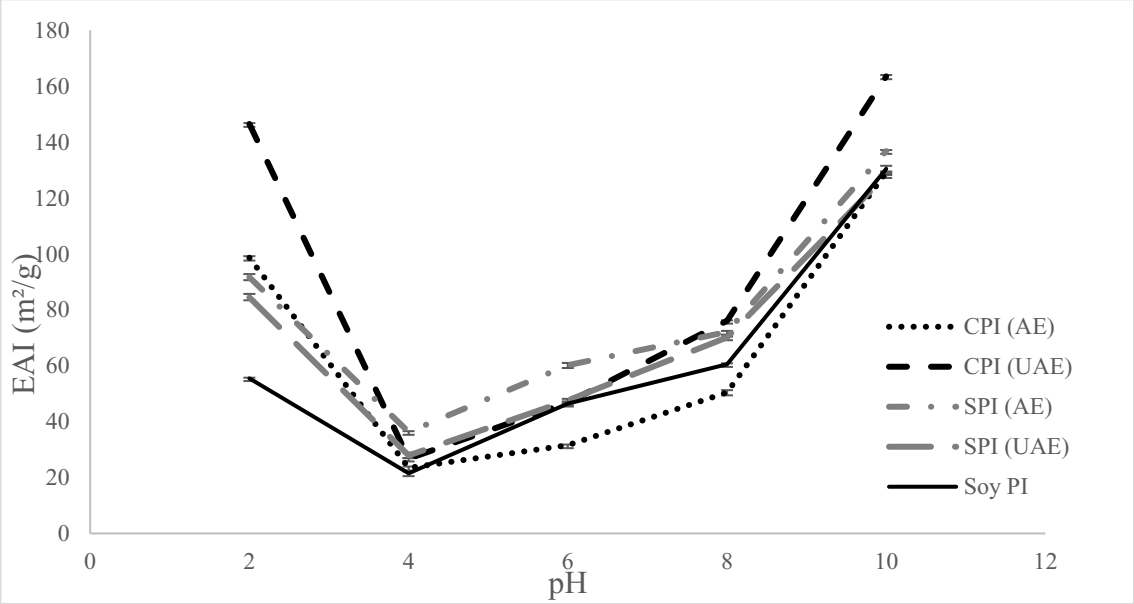


Figure 3.5 Effect of pH on emulsifying ability index (EAI) of camelina protein isolate (CPI) and sophia protein isolate (SPI) prepared by alkaline extraction (AE) and ultrasound-assisted alkali extraction (UAE)

Data presented are means of triplicate analyses ± standard deviation ($p<0.05$)

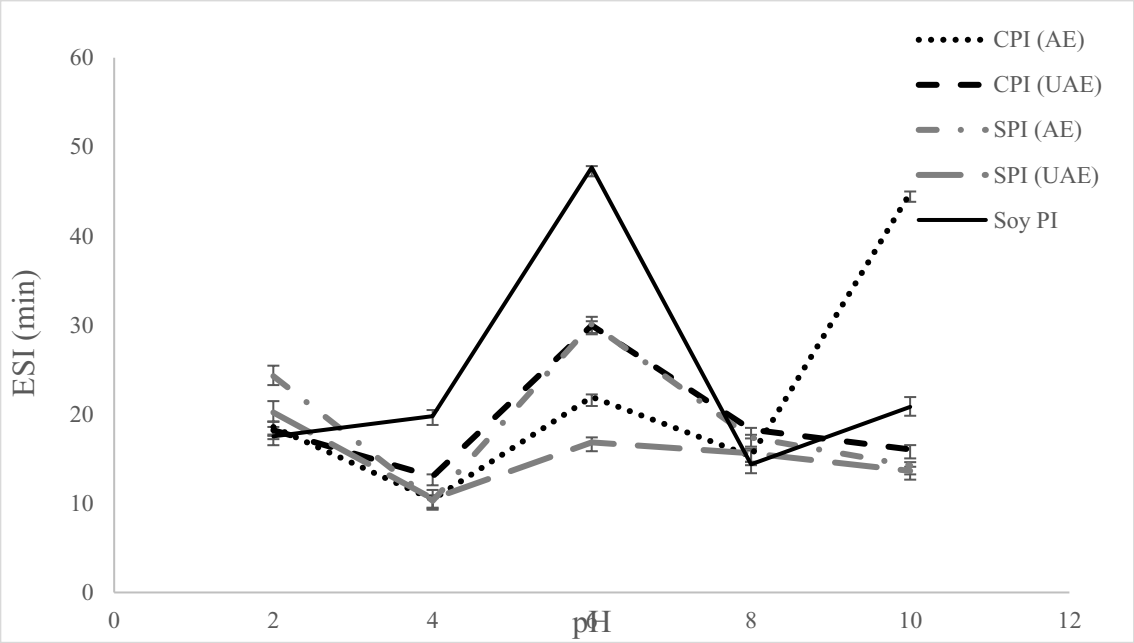


Figure 3.6 Effect of pH on emulsifying stability index (ESI) camelina protein isolate (CPI) and sophia protein isolate (SPI) prepared by alkaline extraction (AE) and ultrasound-assisted alkali extraction (UAE)

Data presented are means of triplicate analyses ± standard deviation ($p<0.05$)

3.5 Conclusion

The ultrasonic-assisted extraction method was more effective than the traditional method for the extraction of proteins from camelina and sophia seed meals. There were no differences in protein subunit bands for all the samples prepared by ultrasonic-assisted extraction. The surface hydrophobicity of CPI and SPI was higher than that of soybean protein isolates. Ultrasonic-assisted extraction significantly improved water holding capacity and oil absorption capacity, emulsifying capacity, and foaming capacity of the CPI and SPI which indicate the potential use of CPI and SPI as a replacement for soybean

protein in food formulations. Further studies on the protein value and digestibility of CPI and SPI are necessary for their application as food ingredients.

3.6 References

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CHAPTER 4

Antioxidant properties of camelina (*Camelina sativa* (L.) Crantz) protein hydrolysates

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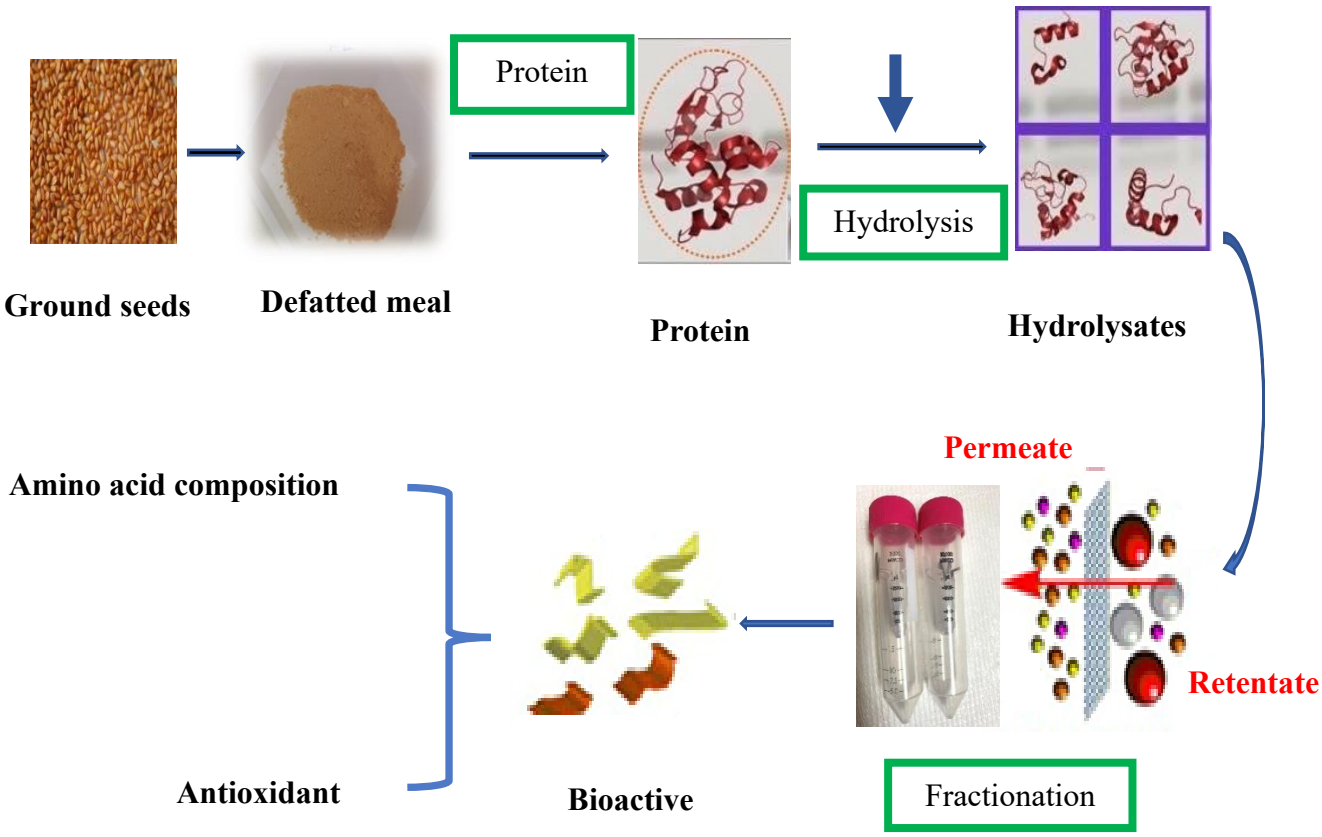
4.1 Abstract

Camelina seed meal was used to produce protein hydrolysates using Alcalase and Flavourzyme. The hydrolysates were then fractionated by employing ultrafiltration membranes (3, 10 kDa). The antioxidant activities of camelina protein hydrolysates and peptide fractions were investigated. The essential amino acid content of camelina protein isolates and hydrolysates was comparable and adequate. All camelina hydrolysates exhibited the highest radical scavenging activity in both DPPH and ABTS assay compared to camelina protein isolates. When comparing the overall DPPH and ABTS radical scavenging activity of peptide fractions, smaller-size peptides (<3 kDa) displayed considerably higher values and hence more potency than larger-sized peptides (>3 kDa). Peptide fractions with 3-10 kDa had better metal chelation and reducing power than those < 3 kDa and > 10 kDa. These findings suggest that camelina protein hydrolysates could be employed as bioactive ingredients in the formulation of functional foods and against oxidative stress.

Keyword: Camelina meal, protein hydrolysates, peptide fractions, antioxidant activity

Graphic abstract



4.2 Introduction

Proteins are macronutrients in food that provide energy and amino acids required for proper human growth and maintenance. Food proteins have a wide range of physicochemical and sensory properties. Protein hydrolysates, particularly containing bioactive peptides, have recently been demonstrated to have a variety of biological functions in addition to their known nutritional benefits. Interests in studying protein hydrolysates and bioactive peptides has intensified in the recent past, due to their multitude of beneficial effects as functional food ingredients, nutraceuticals, or as medicinal (Ambigaipalan et al., 2015; Senadheera et al., 2021).

A wide variety of foods, including animals, plants, and marine products, have been used as sources of bioactive peptides. Production of such products is generally carried out in pursuit of value-added use of underutilized proteins or protein-rich food industry's by-products, and utilization of proteins containing specific peptide sequences or amino acid residues of pharmacological interest (Udenigwe and Aluko, 2012). Seed meals and cakes produced from important oilseed crops are attractive co-products due to their high content of protein and presence of bioactive compounds. Therefore, these could be considered as valuable plant-derived feedstocks for food and non-food applications. In this respect, camelina (*Camelina sativa* (L.) Crantz) meal is gaining attention as an ingredient in functional foods and cosmetic additives in the health sector (Tavarini et al., 2021).

Protein hydrolysates are produced by the cleavage of peptide bonds that can be achieved by enzymatic or chemical processes. Using enzymes to produce protein hydrolysates has gained much attention as a potential method of making functional food

ingredients and nutraceuticals for disease prevention and health promotion (Alashi et al., 2014; Ambigaipalan et al., 2015; Senadheera et al., 2021). Generally, an acceptable food protein source must be chosen based on the physiological properties of products and then treated with a single or multiple specific or nonspecific protease to release peptides of interest. The type of enzyme determines the cleavage pattern of peptide bonds. The principal enzymes employed for peptide production are endo- and exo-proteinases such as trypsin, chymotrypsin, and pepsin (endoproteinase), as well as commercial proteases such as Alcalase and Flavourzyme. Because of their favorable operating conditions, microbial proteases such as Alcalase and Flavourzyme are used in industrial manufacturing (Kristinsson and Rasco, 2000; Udenigwe and Aluko, 2012).

Several studies on the production of hydrolysates from various protein sources including plants and their by-products have been performed such as using hemp seed (Girgih et al., 2011), rapeseed (He et al., 2013), canola meal (Alashi et al., 2014), kidney bean (Mundi and Aluko, 2014), date seed (Ambigaipalan et al., 2015), and mung bean (Carlos et al., 2017), among others. This research line has shown that hydrolysates and/or peptides from them reduce oxidative stress and improve health as well as being able to render certain functional properties to food. More importantly, because of their nutritional and biological qualities, active protein hydrolysates and/or peptides from them can be used as functional food ingredients (Girgih et al., 2011).

Camelina, an ancient oilseed, is also known as Gold-of-pleasure or false flax. It belongs to the Brassicaceae family and is cultivated for its oil, mainly in Europe and in North America. Camelina is a new potential oilseed and a novel source of plant protein

(Ngo and Shahidi, 2021; Tavarini et al., 2021). Although camelina meal is rich in protein (40-45%), its current use is mainly limited to animal and aquaculture feeds (Boyle et al., 2018; Hixson et al., 2016). Furthermore, the nutritional quality of camelina protein is similar to that of canola protein and competes with soy protein for some applications targeting the use of plant proteins (Li et al., 2015).

Compared to its use as an animal feed ingredient, however, camelina meal could be used to manufacture bioactive products with value-added benefits. Some previous studies have focused on using camelina meals to produce protein isolates with the aim to improve food formulation or supplying nutritional benefits (Boyle et al., 2018; Li et al., 2014; Ngo and Shahidi, 2021). Research on the production and the characterization of camelina protein is limited. No study has so far been carried out to produce hydrolysates and bioactive peptides and determine antioxidant properties of camelina protein hydrolysates. To the best of our knowledge, this is the first report on using camelina seeds to manufacture protein hydrolysates using Alcalase and Flavourzyme and investigate their antioxidant potential.

4.3 Materials and methods

4.3.1 Materials

Camelina seeds were used in this study. Camelina seeds were obtained from Linnaeus Plant Sciences INC, Saskatoon, SK, Canada. Alcalase (2.4 AU/g) and Flavourzyme (1000 LAPU/g) were procured from Novozymes, Bagsvaerd, Denmark. All chemicals used were obtained from Fisher Scientific Ltd. (Ottawa, ON, Canada) or Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

4.3.2 Methods

4.3.2.1 Preparation of camelina protein isolates (CPI).

Camelina/Sophia seed samples were defatted with hexane at a 1:5 (w/v) ratio for 5 min in a Waring blender at room temperature. Camelina protein isolates were prepared from defatted camelina meals according to the method described by Ngo and Shahidi (2021). The defatted samples were suspended in distilled water (DW) (3.0%, w/v). The slurry was stirred with a magnetic stirrer for 30 min and the pH was then adjusted to 12 by the addition of a known amount of 2 M NaOH. Subsequently, the suspended sample was placed in an ultrasonic bath (180 W, 40 kHz, 20 min) to extract the proteins. Under similar conditions, the residues were re-extracted two more times with DW. The supernatants were combined, and the pH was adjusted to 4.5 with the addition of 2 M HCl and then centrifuged at 10,000 x g for 30 min at 4ºC to precipitate the protein. The pellets were collected and then washed twice with DW. The precipitated protein was redispersed in DW, and the pH was adjusted to 7.0 with 1 M NaOH. The extracted proteins were freeze-dried and stored at -20ºC for further analyses.

4.3.2.2 Preparation of camelina protein hydrolysates and membrane fractions

Hydrolysis of camelina protein isolates was conducted with Alcalase (0.3 AU/g isolates) and Flavourzyme (50 LAPU/g isolates) under different conditions (Ambigaipalan et al., 2015; Senadheera et al., 2021). The protein isolate (1:4, w/v) was suspended in deionized water. The samples were hydrolysed batchwise with Alcalase (AL, pH 8, 50°C) for 4h, or Flavourzyme (FL, pH 7, 50°C) for 4 h. Enzyme combination treatments were carried out by first hydrolysing with Alcalase for 2 h (pH 8, 50°C), and then adding

Flavourzyme for 2 h (pH 7, 50°C). The pH of the reaction mixture was kept constant throughout the process by adding 4 M NaOH. After digestion, the reactions were terminated by heating the mixture at 90°C for 10 min to inactivate the enzyme. The hydrolysates were collected by centrifugation at 10,000 g for 15 min. A portion of the collected supernatants was freeze-dried and stored at − 20 °C as camelina protein hydrolysate, while the remaining portion was passed through ultrafiltration membranes (Amicon® Ultra) with molecular weight cut-off (MWCO) of 3 and 10 kDa. The ultrafiltration was conducted by first passing the hydrolysate through the 3 kDa membrane. The resulting retentate was then passed through the 10 kDa membrane. The permeate from each MWCO membrane was separated into three peptide fractions as <3, 3–10, and >10 kDa. All permeates were freeze–dried and kept at − 20°C in a freezer until further analysis.

4.3.2.3 ABTS radical cation scavenging assay

The radical scavenging activity of protein hydrolysates was evaluated using the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$) method reported by John and Shahidi (2010) with minor modifications. The samples and reagents were prepared in 0.1 M phosphate buffer (PBS) containing 0.15 M sodium chloride (pH 7.4). $ABTS^{\bullet+}$ solution was prepared by mixing 2.5 mmol 2,2′-azobis-(2-methylpropionamidine) dihydrochloride (AAPH) with 2.5 mmol ABTS stock solution at 1:1(v/v) ratio. The mixture was heated to 60°C for 20 min before being stored at room temperature in the dark. The samples (40 µL,1 mg/mL) were combined with the $ABTS^{\bullet+}$ solution (1.96 mL) and allowed to react for 6 min before reading the absorbance at 734 nm. Similarly, a blank was prepared by replacing the sample with distilled water. A standard

curve was built by using different concentrations of Trolox (0-1000 μM). The decrease in the absorbance at 734 nm after 6 min of the addition of a test compound was used in calculating the results. ABTS radical scavenging activity, represented as μmol of Trolox equivalents (TE) per milligram (mg) of protein hydrolysates, was calculated using the following equation:

ABTS radical scavenging ability (%) =

$$\frac{(\text{Absorbance of the blank} - \text{Absorbance of the sample after 6 min}) \times 100}{\text{Absorbance of the blank}}$$

4.3.2.4 DPPH radical scavenging assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of camelina protein hydrolysates and its peptide fractions was determined using a previously described method (Aluko and Monu, 2003; Chandrasekara and Shahidi, 2011) with slight modifications. Protein hydrolysates solutions (0.5 mg/mL) were prepared in 0.1 M sodium phosphate buffer, pH 7.0, while DPPH (0.3 mM) was prepared in methanol. The samples (50 μL) were mixed with DPPH (250 μL) and placed into a 96-well microplate. The mixture was allowed to stand in the dark at 25ºC for 30 min. Phosphate buffer (pH 7.0) was used as the control. The control (A_c) and samples (A_s) absorbance values were measured at 517 nm. Trolox was used to preparing the standard curve (50–500 μM). DPPH radical scavenging capacity, expressed as μmol of Trolox equivalents (TE) per milligram (mg) of a protein hydrolysate, was calculated using the following equation:

DPPH radical scavenging activity (%) =

$$\frac{(\text{Absorbance of the control} - \text{Absorbance of the sample})}{\text{Absorbance of the control}} \times 100$$

4.3.2.5 Metal chelation activity

Ferrous ion chelating activity was determined according to a previously reported method (Ambigaipalan et al., 2015). Camelina protein hydrolysates (0.5 mg/mL) were prepared in distilled water. The samples (200 µL) were mixed with 1.74 mL of distilled water, 20 µL of $FeCl_2$ (2 mM), and 40 µL of ferrozine (5 mM). The mixture was incubated at room temperature for 10 min, and the absorbance of the solution was read at 562 nm. Distilled water was used as the control. A standard curve was constructed using trisodium salt of ethylenediaminetetraacetic acid (Na_3EDTA). Metal ion chelating ability (%) was calculated using the following equation:

$$\text{Metal chelating activity (\%)} = \left(1 - \frac{\text{Absorbance of the sample}}{\text{Absorbance of the control}}\right) \times 100$$

4.3.2.6 Reducing power

The reducing power of camelina protein hydrolysates was evaluated according to the method described by Cumby et al. (2008) and Ambigaipalan et al. (2015). Camelina protein hydrolysates (0.5 mg/mL) were dissolved in phosphate buffer (0.2 M, pH 6.6). The samples (1 mL) were added 2.5 mL of 1% potassium ferricyanide solution, and the mixture was incubated at 50 °C for 20 min. After that, 10% trichloroacetic acid (2.5 mL) was added to the mixture, which was centrifuged for 10 min at 1,000 g. After centrifugation, 2.5 mL of supernatant was mixed with 2.5 mL of deionized water and 0.5 mL of 0.1% ferric chloride solution. The solution's absorbance was read at 700 nm after being allowed to react

for 10 min. The control had no hydrolysates added, whereas the blank had only protein hydrolysates. A standard curve was constructed using different concentrations (0-1,000 μM) of Trolox, and the reducing power was expressed as μmol of Trolox equivalents (TE) per milligram (mg) of protein.

4.3.2.7 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging capacity was determined using an EPR spectrometric (Bruker E-scan, Bruker Biospin Co., Billericia, MA, USA) method as described by Chandrasekara and Shahidi (2011), with slight modifications. The camelina protein hydrolysates were dissolved in deionized water. The sample (200 μL) was mixed with 10 mM H_2O_2 (200 μL), 17.6 mM 5,5-dimethyl-1-pyrroline N-oxide (DMPO; 400 μL) and 10 mM $FeSO_4$ (200 μL). After 3 min at room temperature, the mixture was injected into the sample cavity of the EPR spectrometer, and their spectra recorded. Deionized water was used as the control. The EPR spectra were recorded, and Trolox (0-50 μM) was used to prepare the standard curve. The hydroxyl radical scavenging capacity, expressed as μmol of histidine or carnosine equivalents per milligram (mg) of a protein hydrolysate, was calculated using the following equation:

Hydroxyl radical scavenging capacity (%) =

$$(1 - \frac{\text{EPR signal intensity for the sample}}{\text{EPR signal intensity for the control}}) \times 100$$

4.3.2.8 Amino acid composition

The amino acid composition of each protein hydrolysates was analysed at the Analytics, Robotics and Chemical Biology Centre (SPARC BioCentre), the Hospital for Sick Children, Toronto, ON, Canada, as previously reported (Mohan and Udenigwe, 2015). Except for tryptophan, cysteine, and methionine, all other amino acids were analyzed using vapor-phase hydrolysis with 6 M HCl and 1% phenol at 110°C for 24 hours. Samples were hydrolyzed with 4.2 M NaOH for 24 hours at 110°C for tryptophan analysis. Before hydrolysis, cysteine was quantified using performic acid oxidation. Norleucine (25 μmol/mL) was used as an internal standard. Following hydrolysis, samples were dried and resuspended in a solution of methanol/water/triethylamine (2:2:1, v/v/v) and vacuum dried for 15 min. This was followed by pre-column derivatization with a derivatizing solution containing methanol/water/triethylamine/phenyl isothiocyanate (PITC) (7:1:1:1, v/v/v). Vacuum-dried derivatized samples were dissolved in sample diluent. Waters ACQUITY UPLC (Milford, MA, USA) was used to analyze diluent aliquots using an UPLC BEH C18 column (0.21 x 10 cm) operated on a modified PICO-TAG gradient at 48°C. The amino acids were measured using a UV detector at 254 nm.

4.3.3 Statistical analysis

All experiments were conducted in triplicates and data were reported as mean ± standard deviation. One-way ANOVA was performed, and means were compared by using Tukey's HSD test ($p < 0.05$), SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

4.4 Results and discussion

4.4.1 ABTS radical cation scavenging assay

The Trolox equivalent antioxidant capacity (TEAC) is used to assess the electron donating potential of antioxidants using the $ABTS^{\bullet+}$ scavenging activity. The blue/green $ABTS^{\bullet+}$ chromophore is produced by the chemical reaction between ABTS and the strong oxidizing agent potassium persulfate, with maximum absorption at 734 nm (Shahidi and Yeo, 2020). This assay is commonly used to evaluate the antioxidant capacity of bioactive compounds (Shahidi and Zhong, 2015).

The ABTS radical scavenging activity of protein hydrolysates and their peptide fractions obtained from camelina protein isolate (CPI) showed significantly higher values ($p<0.05$) when compared to that of camelina protein isolates (Figure 4.1).

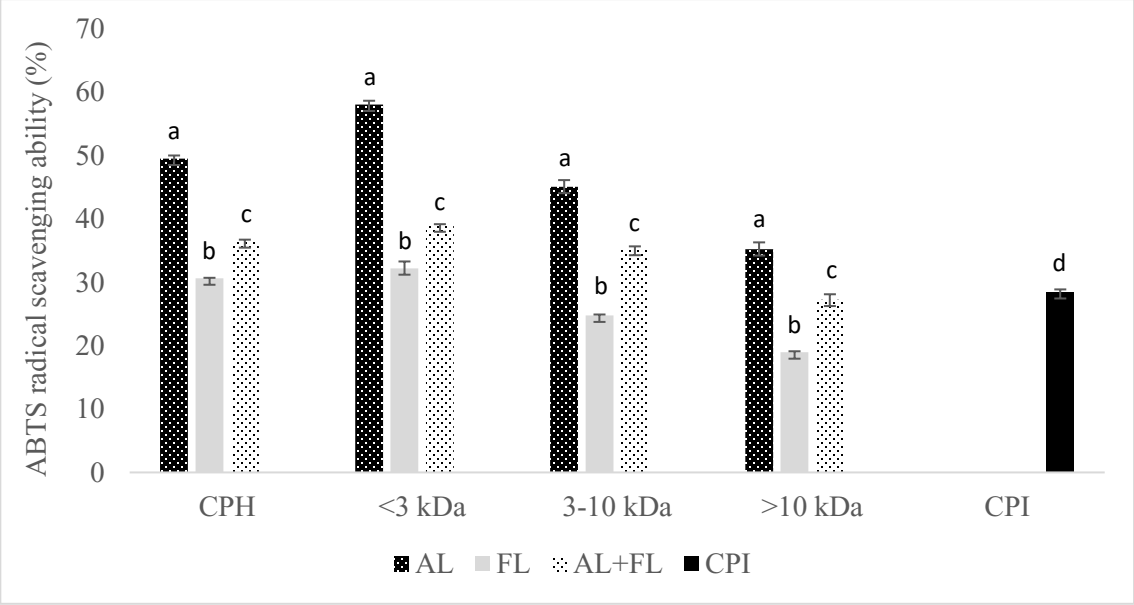


Figure 4.1 ABTS radical scavenging activity of camelina protein isolates (CPI), hydrolysates (CPH) and their peptide fractions prepared by using enzymatic treatment (AL, Alcalase; FL, Flavourzyme; and AL+FL, Mixture of Alcalase and Flavourzyme)

All data represent means of triplicate determinations. Values followed by the same alphabets are not significantly different ($p >0.05$) by Tukey's HSD test.

The considerable difference between the unhydrolyzed and hydrolyzed samples suggests that antioxidant peptides were released from CPI during enzyme digestion, and that these peptides can donate hydrogen atoms to reduce $ABTS^{\bullet+}$ (Alashi et al., 2014). Hydrolysates prepared using Flavourzyme had the lowest ABTS radical scavenging activity (30.6%), while Alcalase hydrolysates showed the highest values (49.49%). Furthermore, Figure 4.1 shows that the Alcalase <3 kDa permeate peptide fraction exhibited the highest ABTS radical scavenging activity (50.02%) while the lowest activity (18.94%) was observed for Flavourzyme >10 kDa. When comparing the overall ABTS radical scavenging activity of peptide fractions, smaller-size peptides (<3 kDa) displayed considerably higher values and hence more potency as $ABTS^{\bullet+}$ scavengers than larger-sized peptides (>3 kDa). The ABTS radical cation activity of camelina peptide fractions were also similar to those of canola peptide fractions (Alashi et al., 2014) and corn meal (Hu et al., 2020).

These findings indicate that the antioxidant activity of peptides could be influenced by their amino acids sequences, the amount of free amino acids present, the degree of hydrolysis and molecular weight of peptides (Ambigaipalan et al., 2015). Alcalase and Flavourzyme have different hydrolysis processes because Alcalase is an endopeptidase, whereas Flavourzyme has both exo- and endopeptidase activity. As a result, the varying antioxidant activity of different enzyme treatments could be related to differences in their composition (Senadheera et al., 2021). Overall, smaller peptides were found to be more effective antioxidants against ABTS radical cation.

4.4.2 DPPH radical scavenging activity

DPPH radical scavenging is a determination of antioxidative properties of compounds as free radical scavengers or hydrogen atom donors and is widely used in the evaluation of peptide, phenolic and food antioxidant capacity (He et al., 2013; Intarasirisawat et al., 2012). There are many parameters that influence the radical scavenging ability of food proteins and their hydrolysates, including their size and the amino acid composition of the peptides, specificity of the protease and DPPH assay conditions (Girgih et al., 2011). Additionally, hydrophobic groups in the hydrolysates increase the peptides' solubility in a non-polar environment, thus providing a greater access to DPPH. This promotes peptides to interact with radicals and trap them so that the chain reaction can be terminated (Senadheera et al., 2021).

Figure 4.2 reveals that the untreated counterparts, CPI showed significantly lower DPPH radical scavenging activities compared to the samples hydrolysed with Alcalase, and Flavourzyme. The study found that camelina protein hydrolysate contains peptides that act as electron donors and free radical scavengers to terminate the chain reaction. These findings lend support to the previously reported study on the radical scavenging activities hydrolysates from various plant proteins (He et al., 2013; Hu et al., 2020; Senadheera et al., 2021). According to the authors, hydrolysates produced by alkaline proteases such Alcalase and Flavourzyme have stronger antioxidant activity than those manufactured with other enzymes as Neutrase, papain, bromelain, and pepsin.

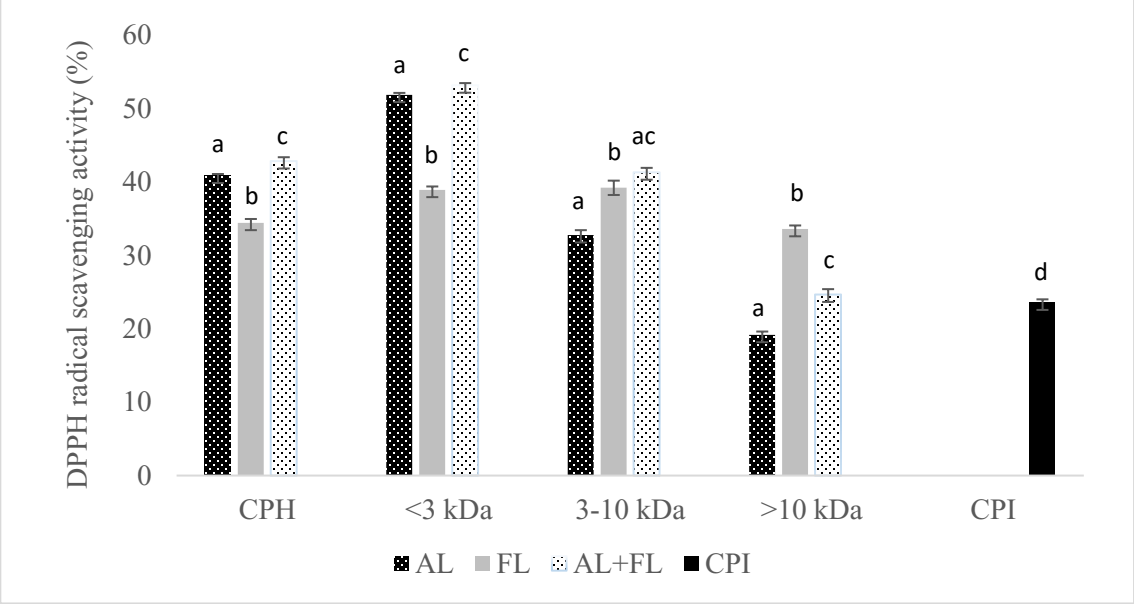


Figure 4.2 DPPH radical scavenging activity of camelina protein isolates (CPI), hydrolysates (CPH) and their peptide fractions prepared by using enzymatic treatment (AL, Alcalase; FL, Flavourzyme; and AL+FL, Mixture of Alcalase and Flavourzyme)

All data represent means of triplicate determinations. Values followed by the same alphabets are not significantly different ($p > 0.05$) by Tukey's HSD test.

The DPPH radical scavenging activity of camelina protein hydrolysates and their peptide fractions (Figure 4.2) show that hydrolysates prepared with Alcalase, and the combination of Alcalase and Flavourzyme, respectively, were the most active against DPPH, while Flavourzyme CPH was the least active. The DPPH inhibitory activity of <3 kDa fractions was significantly higher than activities of 3-10 and >10 kDa. Some researchers have also reported that the DPPH inhibitory activity of peptide fractions depended on the molecular size (Aluko and Monu, 2003; Girgih et al., 2011; Hu et al., 2020).

Furthermore, differences in amino acid compositions, sequence, and structure could explain the difference in antioxidant activity between all treatments (Ambigaipalan et al.,

2015). For instance, Aromatic amino acid residues (Tyr, His, Trp, Phe) can donate hydrogen atoms to electron deficient radicals via resonance stabilization, which improves the radical-scavenging properties of the amino acid residues (Ambigaipalan and Shahidi, 2017). This research indicates that low-molecular-weight peptides have better radical scavenging properties than high-molecular-weight ones, consistent with previous findings for quinoa, hemp seed, and canola protein hydrolysates fractions (Alashi et al., 2014; Aluko and Monu, 2003; Girgih et al., 2011). The trends observed for both ABTS and DPPH radical scavenging activity of the camelina protein hydrolysates and their peptide fractions prepared by using Alcalase followed comparable patterns. The difference between the hydrolysates produced by different enzymes dictates the antioxidant potential of the final products (Cumby et al., 2008). Our research on date camelina protein hydrolysates found that the peptides in the mixture could act as electron donors, inhibiting the radical chain reaction and generating non-radical products.

4.4.3 Metal chelation activity

Iron, a transition metal, is involved in the Fenton reaction, and stimulates lipid oxidation and hence acting as a prooxidant, which eventually leads to degrading hydroperoxide into volatile compounds (Shahidi and Zhong, 2015). Certain phenolic antioxidants and some peptides can form complexes with transition metal ions, thus retarding the oxidation process. This ability is attributed to the presence of amino acid residues including histidine, cystine, tryptophan, aspartate, and glutamate, which have been shown to bind divalent metal ions when exposed on the surface of proteins and polypeptides

(Udenigwe et al., 2016). Figure 4.3 showed the Fe^{2+}chelating effects of the camelina protein hydrolysates and their peptide fractions.

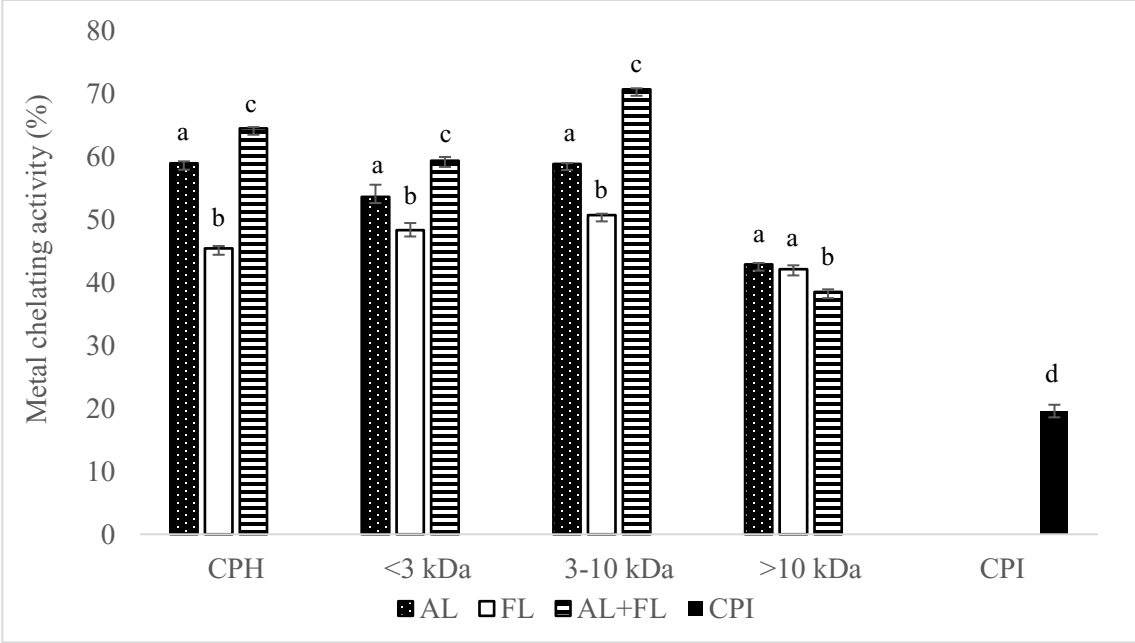


Figure 4.3 Metal chelating activity of camelina protein isolates (CPI), hydrolysates (CPH) and their peptide fractions prepared by using enzymatic treatment (AL, Alcalase; FL, Flavourzyme; and AL+FL, Mixture of Alcalase and Flavourzyme)

All data represent means of triplicate determinations. Values followed by the same alphabets are not significantly different ($p > 0.05$) by Tukey's HSD test.

Clearly, chelation of metal ions by camelina protein hydrolysates treated with the enzyme exhibited significantly higher values than those of their protein isolates. Moreover, ferrous ion chelation of camelina protein hydrolysates varied significantly and followed the order of AL+FL < AL < FL. Interestingly, we observed that the highest metal chelation activity was noted in the 3-10 kDa peptides fraction (70.64%) prepared by the combination of Alcalase and Flavourzyme. The <3 kDa fraction showed low chelating activity compared

with the 3-10 kDa peptides fraction. The highest amount of negatively charged amino acids was found in 3-10 kDa fraction in a prior study, which could have contributed to greater electrostatic attraction for positively charged amino acids (Girgih et al., 2011). Moreover, the discrepancies between the samples may be due to differing charged amino acid side chain residues that can remove transition metal ions. Furthermore, due to its imidazole group, the presence of histidine at the N terminal can be linked to a significant metal ion chelation (Ambigaipalan et al., 2015). These findings suggest that peptide cleavages may promote metal ion binding, eliminating prooxidative metal ions from the system due to the increased concentration of carboxylic groups and amino groups in the side chain of acidic and essential amino acids, respectively.

4.4.4 Reducing power

The ferric reducing antioxidant power assay is widely used to estimate natural antioxidants' ability to donate an electron or a hydrogen atom, and the compounds exhibiting reducing power can reduce the oxidized intermediates of peroxidation (Ambigaipalan et al., 2015). Some research has indicated a correlation between reducing power and antioxidant activity of protein hydrolysate fractions (Girgih et al., 2011; He et al., 2013). Table 4.1 shows the reducing power of camelina protein hydrolysates, and its fractions measured at 700nm with values ranging from 0.65±0.02 to 1.44±0.01 μmol TE/mg of protein.

Table 4.1 Ferric reducing antioxidant power assay (FRAP) of camelina protein hydrolysates and peptide fractions prepared using enzymatic treatment

Proteases	Reducing power (μmol TE/mg of protein)			
	Hydrolysates	<3 kDa	3-10 kDa	>10 kDa
AL	1.39 ± 0.02^{aB}	1.1 ± 0.01^{aC}	1.44 ± 0.01^{aA}	0.82 ± 0.01^{bD}
FL	0.90 ± 0.01^{cB}	0.64 ± 0.01^{cC}	1.20 ± 0.01^{bA}	0.92 ± 0.02^{aB}
AL+FL	1.27 ± 0.01^{bA}	0.77 ± 0.01^{bD}	1.08 ± 0.02^{cB}	0.82 ± 0.01^{bC}

AL, Alcalase; FL, Flavourzyme; AL+FL, Mixture of Alcalase and Flavourzyme
Data presented as mean ± SD (n = 3). Results followed by the same lowercase letter in the column and by the same uppercase letter in the row are not significantly different at $p < 0.05$.

The highest reducing power was found in hydrolysates treated with Alcalase. These findings match those of ABTS and DPPH radical scavenging activities. In addition, a higher reducing power was observed for 3-10 kDa fraction for all the three types of hydrolysates, varying from 1.07±0.02 to 1.44±0.01 μmol TE/mg of hydrolysate fraction. There was an increase in reducing power of the camelina protein fractions with increasing peptide size (3–10 kDa fractions were better than < 3 kDa fraction), indicating additive effects of active groups within the peptide molecules. The reducing power of the 3-10 kDa fraction was similar to the trend for the metal chelating effects of the corresponding samples. The camelina protein hydrolysates showed considerably higher values than both the <3 kDa and >10 kDa fractions, implying that the presence of 3-10 kDa peptides contributed to the activity of the camelina protein hydrolysates.

The current results are supported by previous findings that ferric reducing antioxidant power was directly influenced by the type of protease used for hydrolysis (Ambigaipalan et al., 2015; Udenigwe et al., 2016). The presence of hydrophobic amino acids or peptides that can react with free radicals to form more stable products, according to the authors, may be responsible for the differences in the activity. In addition, the presence of amino acids leucine, isoleucine, histidine, methionine, tyrosine, lysine, and tryptophan may have contributed to the reducing power activity observed for protein hydrolysates (Girgih et al., 2011). According to Udenigwe et al. (2016), electron donation by amino acid residues, including the sulfhydryl group of cysteine, also contributes to the reducing capacity of peptides. As a result, the presence of sulfhydryl groups or their oxidized forms directly impacts the reducing ability of protein hydrolysates.

4.4.5 Hydroxyl radical scavenging activity

Hydroxyl radicals are considered the most reactive free radicals in biological systems, reacting with biomolecules such as amino acids, proteins DNA, and membrane lipids to induce severe damages to cells through oxidative stress (Girgih et al., 2011; Shahidi and Yeo, 2020). As a result, removing excess level hydroxyl radicals is recognized to be one of the most effective defense approaches in the prevention of a variety of cellular diseases, including cancer, cardiovascular disease, and diabetes, among others (Girgih et al., 2011). The hydroxyl radical scavenging activity of camelina protein hydrolysates and their fractions is shown in Table 4.2 and the values varied between 1.57 and 3.8 μmol histidine equivalents /mg of protein.

Table 4.2 Hydroxyl radical scavenging of camelina protein hydrolysates and peptide fractions prepared using enzymatic treatment
AL, Alcalase; FL, Flavourzyme; AL+FL, Mixture of Alcalase and Flavourzyme

Protease	μmol of Histidine/mg protein				Hydroxyl radical scavenging activity (%)			
	Hydrolysates	<3 kDa	3-10 kDa	>10 kDa	Hydrolysates	<3 kDa	3-10 kDa	>10 kDa
AL	3.21±0.03 ^{aB}	3.80±0.03 ^{aA}	2.90±0.02 ^{aC}	1.93±0.09 ^{aD}	52.11±0.38 ^{aB}	59.00±0.39 ^{aA}	48.41±0.27 ^{aC}	37.06±1.04 ^{aD}
FL	2.50±0.06 ^{cB}	2.92±0.04 ^{cA}	2.27±0.05 ^{cC}	1.57±0.05 ^{bD}	43.73±0.72 ^{cB}	48.65±0.44 ^{bA}	41.05±0.54 ^{cC}	32.79±0.53 ^{bD}
AL+FL	2.71±0.06 ^{bB}	3.25±0.04 ^{bA}	2.77±0.09 ^{bB}	2.03±0.05 ^{aC}	46.16±0.75 ^{bB}	52.56±0.46 ^{cA}	46.90±1.06 ^{bB}	38.28±0.63 ^{aC}

Data presented as mean ± SD (n = 3). Results followed by the same lowercase letter in the column and by the same uppercase letter in the row are not significantly different at $p < 0.05$

Hydrolysates prepared using Alcalase had a higher hydroxyl radical scavenging ability compared to all other enzyme treatments in each group. The hydroxyl radical scavenging activity in terms of percentage ranged from 33 to 59 %. Similar observations were made by Ambigaipalan et al. (2015) for hydrolysates prepared from date seed. In addition, Alcalase hydrolysed <3 kDa fraction had the highest value amongst all tested samples. This indicates that that the lower molecular weight peptides had a greater the hydroxyl radical scavenging activity. The camelina seed peptides are effective scavengers of hydroxyl radical when compared to those of the hemp seed (Girgih et al., 2011). According to Cumby et al. (2008), the radical scavenging activity of peptides or protein hydrolysates is related to the hydrogen donor activity of the hydroxyl groups of aromatic amino acid residues (tyrosine, histidine, tryptophan, and phenylalanine). Radical scavenging activity of these aromatic amino acid residues improves through resonance stabilization. Thus, the presence or lack of certain amino acids in peptides, as well as their position in the peptide sequence, has an impact on antioxidant activity.

4.4.6 Amino acid composition

The amino acid composition of protein hydrolysates significantly impacts their antioxidant activity. The presence of Tyr, Met, His, and Lys has been demonstrated to contribute to the potency of antioxidant peptides (Alashi et al., 2014; He et al., 2013). Camelina protein isolates and hydrolysates digested with Alcalase were further analysed for their amino acid profiles. Table 4.3 shows that the hydrolyzation process had no negative impact on the hydrolysates' amino acid composition. Such finding lends support to the previous finding on canola meal (Alashi et al., 2014).

Table 4.3 Amino acid composition of camelina protein isolates and hydrolysates (Alcalase) (g/100g product)

Essential amino acid (EAA)	Camelina		WHO/FAO
	Protein isolates	Protein hydrolysates	(1991)
Histidine (His)	2.43	2.12	1.6
Isoleucine (Ile)	3.54	2.88	1.3
Leucine (Leu)	6.19	4.94	1.9
Lysine (Lys)	3.34	2.31	1.6
Methionine (Met)	1.78	1.54	1.7
Phenylalanine (Phe)	4.19	3.27	
Threonine (Thr)	3.45	2.81	0.9
Tryptophan (Trp)	0.91	1.37	0.5
Valine (Val)	4.64	3.86	1.3
Non EAA (NEEA)			
Alanine (Ala)	3.18	2.78	
Arginine (Arg)	8.61	7.04	
Aspartic acid +Asparagine (Asp+Asn)	7.20	6.38	
Cystine (Cys)	1.38	1.38	
Glutamic acid +Glutamine (Glu + Gln)	15.43	12.69	
Glycine (Gly)	4.12	3.22	
Proline (Pro)	4.22	3.45	
Serine (Ser)	3.81	3.08	
Tyrosine (Tyr)	2.97	2.42	
Phenylalanine+ Tyrosine (Phe + Tyr)	7.16	3.27	1.9

In addition, the amino acid composition of this study correspond with the previous study on the camelina seeds (Russo and Reggiani, 2015). In camelina protein samples, leucine was the most abundant essential amino acid (EAA), while tryptophan, histidine and

methionine contributed a relatively low amount. During the analysis procedure, sensitive amino acids such as methionine and tryptophan may be impacted (Senadheera et al., 2021). Moreover, camelina protein hydrolysates' amino acid profiles satisfy the World Health Organization/Dietary and Agriculture Organization (WHO/FAO) 1991 recommendations for the quantity of most essential amino acids in food products. According to the present work, camelina protein hydrolysates can be employed as a good source of dietary protein.

Table 4.3 shows that the amino acid composition of camelina protein hydrolysates was rich in Glu, Asp, Arg, and Leu. According to reported studies, negatively charged amino acids such as Glu and Asp have potent antioxidant properties due to being a rich source of electrons that can be donated to free radicals. The high quantities of lysine and leucine found in camelina protein hydrolysates could enhance superoxide radical scavenging (Udenigwe et al., 2016). Furthermore, hydrophobic amino acids including tyrosine, methionine, histidine, cysteine, and tryptophan can positively impact the antioxidant activity. For instance, tyrosine residue could serve as a potent hydrogen donor, and histidine can be credited with strong radical scavenging activity due to the decomposition of its imidazole ring (Senadheera et al., 2021). Therefore, it has been suggested that the amino acid profile can be used to predict the antioxidant activity of protein hydrolysates and peptides (Udenigwe et al., 2016).

4.5 Conclusion

The camelina protein hydrolysates and their fractions was found to possess potent antioxidant effects *in vitro*. Furthermore, they had the ability to scavenge free radicals which was mainly dictated by the peptide size. Thus, low molecular weight peptides had a stronger activity. The <3 kDa peptide fractions were generally the most effective scavengers of free

radicals (DPPH, ABTS and hydroxyl radicals). The 3-10 kDa fractions had better metal chelation and ferric reducing power than the < 3, and > 10 kDa fractions. According to our findings, camelina seeds could be a good source of antioxidant hydrolysates and peptides. Hydrolysates may also serve as a natural value-added component in the formulation of functional foods and nutraceutical, and a good source of dietary protein due to their rich profile of essential amino acids. However, more research is needed to determine the amino acid sequence essential for antioxidant and other biological functions.

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CHAPTER 5

Antioxidant properties and prediction of bioactive peptides produced from camelina (*Camelina sativa* (L.) Crantz) and flixweed (sophia, *Descurainis sophia* L.) seed meals

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5.1 Abstract

Flixweed (sophia) seed meal and camelina were used to produce protein hydrolysates using Alcalase and Flavourzyme. The antioxidant activities of sophia/camelina protein hydrolysates were investigated. In addition, the prediction of bioactive peptides was achieved by employing bioinformatics methods. The aim of this research was to focus on utilizing sophia and camelina protein hydrolysates for releasing potent antioxidative and angiotensin-converting enzyme (ACE) inhibitory peptides, and dipeptidyl peptidase IV (DPP IV) inhibitors using various *in silico* approaches (Peptideranker, BIOPEP, Toxinpred, SwissADME, AlgPred). The hydrolysates exhibited the highest radical scavenging activity against both DPPH and ABTS radicals compared to protein isolates. Besides, the highest capacity for scavenging hydroxyl radicals and oxygen radical absorbance capacity was observed in protein hydrolysates derived from sophia and camelina using the Alcalase enzyme. In addition, hydrolysates inhibited LDL cholesterol oxidation, and hydroxyl and peroxyl radical-induced DNA scission. Interestingly, all digestive resistant peptides were nontoxic and had desirable drug-like properties. This indicates that sophia/camelina protein hydrolysates may have the potential for use in various food formulations and nutraceuticals.

Keyword: Bioactive peptides, bioinformatics, BIOPEP-UWM database, antioxidant activity, angiotensin-converting enzyme (ACE) inhibitory peptides, dipeptidyl peptidase IV (DPP IV) inhibitory peptide.

5.2 Introduction

Natural plant-derived proteins, as opposed to those from animal sources, are currently attracting a lot of attention due to their sustainable availability and economic attraction (Deng et al., 2019). Food-derived protein hydrolysates and their bioactive peptides are a new type of potentially active ingredient that can be used to develop nutraceuticals and functional food because they are safe and have a wide range of possible health benefits (Udenigwe and Aluko, 2012; Samtiya et al., 2021). Thus, diversified sources of plant proteins increasingly address the global need for ingredients in the food industry. Furthermore, identification of bioactive peptides encoded in oilseed processing waste has received much attention in recent years. It has also been highlighted as a viable biorefinery strategy for addressing the detrimental effects of unregulated waste disposal on the environment (Udenigwe and Aluko, 2012). In this regard, camelina and sophia seed meals have emerged as potential functional food ingredients due to their nutritional value and beneficial health effect (Radman et al., 2018; Ngo and Shahidi, 2021a).

Protein hydrolysates are prepared by cleaving the peptide bonds, either enzymatically or chemically. The use of enzyme hydrolysis to manufacture protein hydrolysates has received much attention to create functional food ingredients and nutraceuticals for reducing disease risk and promoting health (Ambigaipalan et al., 2015; Ngo and Shahidi, 2021b). Many researchers have reported that peptides prepared by enzymatic hydrolysis from plant sources such as soybean, flaxseed, quinoa, and date seed have various physiological and biological properties such as antioxidative, and angiotensin-I-converting enzyme (ACE) inhibition, and dipeptidyl peptidase IV (DPP IV) inhibition (Ambigaipalan et al., 2015; Mune

et al., 2018; Wang et al., 2019; Mudgil et al., 2020; Langyan et al., 2021). According to the World Health Organization (WHO), the proportion of individuals suffering from cardiovascular diseases (CVD) and type 2 diabetes (T2D) is growing tremendously. However, some synthetic therapeutic medications have been produced to treat these diseases, many of them are considered harmful due to adverse effects such as inflammatory responses, taste disturbances, nausea, headaches, and allergic reactions (Mune et al., 2018; Samtiya et al., 2021). Furthermore, oxidative damage is linked to several pathogenic problems, including diabetes, cardiovascular disease, cancer, and other chronic and inflammatory diseases (Mune et al., 2018). For human disease prevention and management, natural antioxidants such as plant protein hydrolysates have arisen as an alternative to chemotherapy, as well as a strategy to maintain a healthy lifestyle.

Traditional methods have been employed for the discovery and creation of novel bioactive peptides, which include numerous processes for the separation, purification, and identification of bioactive peptides. However, this method is time-consuming and laborious. Currently, these problems have been solved by using bioinformatic methods that save time and work better than traditional methods (Udenigwe, 2014). This method is designed to investigate bioactive peptides that are already present in protein sequences. It also includes details on the specificities and types of proteases used to generate bioactive peptide sequences (Langyan et al., 2021). Besides, mass spectrometry (MS)-based proteomics, a relatively recent technology, has been widely adopted and applied in the development of protein analysis to detect proteins and peptides from dietary sources (Panjaitan et al., 2018). After peptides are identified, the sequence can be further observed using bioinformatic tools,

including UniProtKB, SwissProt, TrEMBL, BIOPEP, and PepBank, to evaluate the potential of proteins as precursors of bioactive peptides (Udenigwe, 2014; Agyei et al., 2018). Limited number of studies have reported the use of camelina and sophia meals as precursor for bioactive peptide. No study has so far been carried out to explore drug likeliness of camelina and sophia meals derived bioactive peptides. To the best of our knowledge, this is the first time that camelina and sophia meal seeds have been employed to produce protein hydrolysates with Alcalase and Flavourzyme and to evaluate their antioxidant potential. This is also the first study to use *in silico* techniques to assess the antioxidative, ACE inhibitory, and DPP IV inhibitory activities of hydrolysates derived from camelina and sophia meals. The *in silico* techniques like PeptideRanker, BIOPEP, ToxinPred, and SwissADME, allergy FP tools to find and screen these potential activities of camelina/sophia peptides to assess their toxicity, physicochemical characteristics, and drug likeliness were among variables tested.

5.3 Materials and methods

5.3.1 Materials

Camelina and sophia seeds were used in this study. Camelina seeds were obtained from Linnaeus plant sciences INC, Saskatoon, SK, Canada and sophia seeds were procured from Daghdagh Abad, near Hamedan city in Iran, and were purchased from the Tavazo store in Toronto, ON, Canada. Alcalase (2.4 AU/ g) and Flavourzyme (1000 LAPU/g) were purchased from Novozymes, Bagsvaerd, Denmark. All chemicals used were obtained from Fisher Scientific Ltd. (Ottawa, ON, Canada) or Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

5.3.2 Methods

5.3.2.1 Preparation of camelina/sophia protein isolates.

Camelina/sophia protein isolates was procured from defatted camelina/sophia meals according to the method described by Ngo and Shahidi (2021a). The defatted samples were suspended in distilled water (DW) (3.0%, w/v). The mixture was stirred with a magnetic stirrer for 30 min and the pH was then adjusted to 12 by the addition of a known amount of 2 M NaOH. In the next step, the suspended sample was placed in an ultrasound bath (180 W, 40 kHz, 20 min) to extract the proteins. Under similar conditions, the residues were re-extracted two more times with distilled water (DW). The supernatants were combined, and the pH was adjusted to 4.5 with the addition of 2 M HCl and then centrifuged at 10,000 g for 30 min at 4ºC to precipitate the protein. The pellets were collected and then washed twice with DW. The precipitated protein was redispersed in DW and the pH was adjusted to 7.0 with 1 M NaOH. The extracted proteins were freeze-dried and stored at -20ºC for subsequent analyses.

5.3.2.2 Preparation of camelina/sophia protein hydrolysates

Hydrolysis of camelina/sophia protein isolates was conducted with Alcalase (0.3 AU/g) and Flavourzyme (50 LAPU/g) under different conditions (Ambigaipalan et al., 2015; Senadheera et al., 2021). The samples were hydrolysed batchwise with Alcalase (AL, pH 8, 50°C) for 4h, and Flavourzyme (FL, pH 7, 50°C) for 4 h. Enzyme combination treatments were carried out by first hydrolysing with Alcalase for 2h (pH 8, 50°C), then adding Flavourzyme for 2 h (pH 7, 50°C). The pH of the reaction mixture was kept constant throughout the process by adding 4 M NaOH. The digestion reactions were terminated by

enzyme inactivation via heating the mixture at 90°C for 10 min. The hydrolysates were collected by centrifugation at 10,000 g for 15 min.

5.3.2.3 DPPH radical scavenging assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of protein hydrolysates and their peptide fractions was determined using a previously described method (Chandrasekara and Shahidi, 2011; Aluko and Monu, 2003) with slight modifications. Protein hydrolysates solutions (0.5 mg/mL) were prepared in 0.1 M sodium phosphate buffer, pH 7.0, while DPPH (0.3 mM) was prepared in methanol. The samples (50 µL) were mixed with DPPH (250 µL) and placed into a 96-well microplate. The mixture was kept in the dark at 25ºC for 30 min. Buffer was used as the control. The absorbance of the control (Ac) and samples (As) was measured at 517 nm. Trolox was used to prepare the standard curve (50–500 µM). DPPH radical scavenging activity (%), expressed as micromoles (µM) of trolox equivalents (TE) per milligram of a protein hydrolysate, was calculated using the following equation:

DPPH radical scavenging activity (%) =

$$\frac{(\text{Absorbance of the control-Absorbance of the sample})}{\text{Absorbance of the control}} \times 100$$

5.3.2.4 ABTS radical cation scavenging assay

The radical scavenging activity of protein hydrolysates was evaluated using the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$) with minor modifications (Ambigaipalan et al., 2015). The samples and reagents were prepared in 0.1 M phosphate buffer (PBS) containing 0.15 M sodium chloride (pH 7.4). $ABTS^{\bullet+}$ solution was

prepared by mixing 2.5 mM 2,2′-azobis-(2-methylpropionamidine) dihydrochloride (AAPH) with 2.5 mM ABTS stock solution at 1:1 (v/v) ratio. The mixture was heated to 60°C for 20 min before being stored at room temperature in the dark. The samples (40 µL,1 mg/mL) were combined with the $ABTS^{\bullet+}$ solution (1.96 mL) and allowed to react for 6 min before reading the absorbance at 734 nm. Similarly, a blank was prepared by replacing the sample with distilled water. A standard curve was built by using different concentrations of trolox (0–1,000 µM). The decrease in the absorbance at 734 nm after 6 min of the addition of a test compound was used in calculating the results. ABTS radical scavenging activity, represented as µmol of Trolox equivalents (TE) per milligram (mg) of protein hydrolysates, was calculated using the following equation:

ABTS radical scavenging ability (%) =

$$\frac{(\text{Absorbance of the blank} - \text{Absorbance of the sample after 6 min})}{\text{Absorbance of the blank}} \times 100$$

5.3.2.5 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging capacity was determined using an EPR spectrometric (Bruker E-scan, Bruker Biospin Co., Billericia, MA, USA) (Ambigaipalan et al., 2015). The protein hydrolysates were dissolved in deionized water. The sample (200 µL) was mixed with H_2O_2 (10 mM, 200 µL), 5,5-dimethyl-1-pyrroline *N*-oxide (17.6 mM DMPO; 400 µL) and $FeSO_4$ (10 mM, 200 µL). The mixtures incubated for 3 min at room temperature, and then injected into the sample cavity of the EPR spectrometer, and their spectra recorded. Deionized water was as the control. The EPR spectra were read, and Histidine (0.1-2.0

mg/mL) was used as the standard curve. The hydroxyl radical scavenging capacity, expressed as micromoles (μM) of histidine equivalents per milligram of a protein hydrolysate.

5.3.2.6 Oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed using a Fluostar Optima plate reader (BMG Labtech, Durham, NC, USA) and the reported method with minor modifications was used (Ambigaipalan et al., 2015). All chemicals and materials were diluted in a 75 mM/L phosphate buffer (pH 7.0) prior to the experiment. Camelina/sophia protein hydrolysates (1mg/mL, 20 μL) and 200 μL of fluorescein (0.11 μM in PBS) were placed on in a Costar® 3695 flat bottomed 96 well black microplates (Corning Incorporated, Corning, NY, USA). The mixture was pre-heated for 15 min at 37°C in the built-in incubator. The equipment was programmed to inject 75 μL of AAPH (17.2 mg/mL in PBS) at the end of incubation during the first cycle. The changes of fluorescence were read every minute for 25 cycles and the time of each cycle was 210 s. Excitation and emission wavelengths were 485 and 520 nm, respectively. ORAC values of protein hydrolysates were expressed as micromoles of trolox equivalents per milligram of protein using the trolox standard curve (6.25–50 μM).

5.3.2.7 Metal chelation activity

Ferrous ion chelating activity was determined according to the previously reported method (Ambigaipalan et al., 2015; Ngo and Shahidi, 2021b). Camelina/sophia protein hydrolysates (0.5 mg/mL) were prepared in distilled water. The samples (200 μL) were mixed with 1.74 mL of distilled water, 20 μL of $FeCl_2$ (2 mM), and 40 μL of ferrozine (5 mM). The mixture was incubated at room temperature for 10 min, and the absorbance of the solution was read 562 nm. Distilled water was used as the control. A standard curve was constructed

using trisodium salt of ethylenediaminetetraacetic acid (Na_3EDTA). Metal ion chelating ability (%) was calculated using the following equation:

$$\text{Metal chelating activity (\%)}=(1-\frac{\text{Absorbance of the sample}}{\text{Absorbance of the control}})\times 100$$

5.3.2.8 Cupric ion-induced human low-density lipoprotein (LDL) peroxidation

The inhibitory activity of camelina/sophia protein hydrolysates against copper-induced LDL cholesterol oxidation was measured according to the reported method (Ambigaipalan et al., 2015). LDL (5 mg/mL) was dialyzed in 10 mM phosphate buffer (pH 7.4, 0.15 M NaCl) using a dialysis tube with a molecular weight cut-off of 12−14 kDa (Fisher Scientific, Nepean, ON, Canada) at 4°C under a nitrogen blanket in the dark for 12 h. Diluted LDL cholesterol (0.04 mg LDL/mL) was mixed with protein hydrolysate solutions (0.1 mg/mL). The positive control used was carnosine. The reaction was initiated by adding 0.1 mL of 100 μM solution of $CuSO_4$. A blank containing only sample without LDL or $CuSO_4$ was prepared for each test compound. After incubation of the reaction mixture at 37 ℃ for 12 h, the conjugated dienes formed was recorded at 234 nm using a diode array spectrophotometer (Agilent, Palo Alto, CA, USA). The inhibitory effect of tested samples was expressed as percentage inhibition of conjugated diene formation according to the following equation:

$$\text{Inhibition (\%)} = ((A_{control} - A_{sample})/(A_{control}-A_{native})) \times 100$$

$A_{control} = (A°_0 - A°_t)$

$A_{sample} = (A_0 - A_t)$

where $A_{control}$ is the absorbance of LDL + $CuSO_4$ + PBS, A_{sample} is the absorbance of LDL + $CuSO_4$ + sample/standard, and A_{native} is the absorbance of LDL + PBS. A_0 and A_t are absorbance values for test samples measured at zero time and at time t after incubation, respectively, whereas $A°_0$ and $A°_t$ are corrected absorbance values for control at time zero and at time t after incubation, respectively.

5.3.2.9 Inhibition of peroxyl and hydroxyl radical-induced supercoiled DNA strand scission

Inhibitory activity of protein hydrolysates prepared from camelina/sophia isolates against DNA strand scission induced by peroxyl and hydroxyl radical was determined according to previous studies, with some modifications (Ambigaipalan et al., 2015). Protein hydrolysates and DNA (50 µg/mL) were dissolved in 10 mM phosphate buffer (PBS, pH 7.4). Agarose gel (50 mL, 0,7%) was first prepared in Tris−acetic acid−EDTA (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.5), and then SYBR safe (5 µL) was added as a gel stain. Protein hydrolysates (0.1 mg/mL, 2 µL) was mixed with PBS (2 µL), pBR 322 (2 µL), and 4 µL of 7 mM 2,2′-azobis (2-methylpropanimidamide dihydrochloride (AAPH) to test their inhibitory activity against peroxyl radicals induced oxidation. Additionally, to assess hydroxyl radical induced oxidation, protein hydrolysates (6 mg/mL, 2 µL) was treated with PBS (2 µL), pBR 322 (2 µL), and $FeSO_4$ (0.5 mM, 2µL), H_2O_2 (0.5 mM, 2 µL). All samples were incubated at 37 °C for 1 h in the dark. A blank (DNA only) and a control (DNA + free radicals), a positive control with carnosine (0.1 mg/mL) were prepared for both assays. After the incubation, 1 µL of dye (0.25% bromophenol blue, 0.25% xylene cyanol, and 50% glycerol) was added, and then 10 µL of the mixture was loaded onto the prepared agarose

gel. Gel electrophoresis was performed at 80 V for 90-120 min in TAE buffer using a horizontal submarine gel electrophoresis apparatus (Owl Separation Systems Inc., Portsmouth, NH, USA). The bands were visualised by Alpha-Imager gel documentation system (Cell Biosciences, Santa Clara, CA, USA) under trans-illumination of UV light and data processing was achieved by the Gel Analyzer 19.1 software. The inhibition of supercoiled DNA strand oxidation (%) was calculated according to the following equation.

$$\text{Inhibition (\%)} = (DNA_{samples}/DNA_{blank})*100$$

5.3.2.10 Amino acid composition

The amino acid composition of each protein hydrolysate was determined at the Analytics, Robotics and Chemical Biology Centre (SPARC BioCentre), the Hospital for Sick Children, Toronto, ON, Canada, as a reported method (Mohan and Udenigwe, 2015). Except for tryptophan, cysteine, and methionine, all other amino acids were analyzed using vapor-phase hydrolysis with 6 M HCl and 1% phenol at 110°C for 24 hours. Samples were hydrolyzed with 4.2 M NaOH for 24 hours at 110°C for tryptophan analysis. Before hydrolysis, cysteine was quantified using performic acid oxidation. Norleucine (25 μmol/mL) was used as an internal standard. Following hydrolysis, samples were dried and resuspended in a solution of methanol/water/triethylamine (2:2:1, v/v/v) and vacuum dried for 15 min. This was followed by pre-column derivatization using methanol/water/triethylamine/phenyl isothiocyanate (PITC) (7:1:1:1, v/v/v/v). Vacuum-dried derivatized samples were dissolved in sample diluent. Waters ACQUITY UPLC (Milford, MA, USA) was used to analyze diluent aliquots using an UPLC BEH C18 column

(0.21 × 10 cm) operated on a modified PICO-TAG gradient at 48°C. The amino acids were measured using a UV detector at 254 nm.

5.3.2.11 Simulated gastrointestinal digestion

LC-MS/MS analysis

LC-MS/MS analysis was conducted at the Analytics, Robotics and Chemical Biology Centre (SPARC BioCentre), The Hospital for Sick Children, Toronto, ON, Canada, using Q_Exactive Orbitrap analyzer outfitted with a nanospray source and EASY-nLC nano-LC system (Thermo Fisher, San Jose, CA, USA), as reported elsewhere (Udenigwe et al., 2016). Data analysis was performed using PEAKS+ software (Bioinformatic Solutions, Waterloo, ON, Canada).

Peptide ranking

PepRank (http://bioware.ucd.ie/compass/biowareweb/) was used to screen peptides for their potential bioactivity which predicts the probability (between 0 and 1) of the peptide being bioactive. To limit the number of false positives, a threshold of 0.8 was selected (Mooney et al., 2012). The most potent camelina/ sophia-derived bioactive peptides were selected for further analysis.

Prediction of potential biological activity profile

Selected peptides were investigated for their bioactive properties, namely antioxidant and antihypertensive activities and dipeptidyl peptidase IV inhibitory effect using the "profiles of potential biological activity" option in BIOPEP-UWMTM data base (http://www.uwm.edu.pl/biochemia/index.php/en/biopep).

Prediction of the bioactivities of the released peptides after simulated digestion

The identified sequences bioactive peptides were subjected to *in silico* hydrolysis by employing the enzyme(s) action feature of BIOPEP-UWMTM using pepsin (EC 3.4.23.1), trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) as representative digestive enzymes to mimic the actual *in vivo* digestion. After that, the option to "search for active fragments" was chosen to get a list of peptides with potential activities such as antioxidant and antihypertensive activities and dipeptidyl peptidase IV inhibitory effect (Panjaitan et al., 2018; Ji et al., 2020).

Toxicity and allergenicity prediction of camelina/ sophia bioactive peptides released after simulated digestion

One of the significant barriers to the sustainable utilization of camelina/sophia proteins for the development of nutraceuticals and functional food ingredients is their toxicity and allergenicity. Therefore, the potential toxicity and allergenicity of the peptides released after simulated digestive proteolysis were investigated using ToxinPred online tool (https:// webs.iiitd.edu.in/raghava/toxinpred/index.html) (Gupta et al., 2013) and allergen FP v.1.0 tool (http://www.ddg-pharmfac.net/AllergenFP), respectively (Dimitrov et al., 2014).

***In silico* physicochemical properties and drug-likeness of released peptides after simulated digestion**

In silico evaluation of drug-likeliness of identified camelina/sophia derived-peptides was investigated based on absorption, distribution, metabolism and excretion parameters

using SwissADME tool (http://www.swissadme.ch/index.php#) as previously described (Daina et al., 2017).

5.3.3 Statistical analysis

All experiments were conducted in triplicates and data were reported as mean ± standard deviation. One-way ANOVA was performed, and means were compared by using Tukey's HSD test ($p < 0.05$), SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

5.4 Results and discussion

5.4.1 ABTS and DPPH radical scavenging activities

The oxidative damage that occurs to the human body is caused by reactive oxygen species (ROS), namely superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), peroxyl radicals ($ROO^{\cdot}$), hydroxyl radicals ($HO^{\cdot}$), and peroxynitrite ($ONOO^{-}$). DPPH and ABTS are commonly used to evaluate the antioxidant capacity of bioactive compounds such as peptides and phenolic compounds (He et al., 2013; Shahidi and Zhong, 2015; Shahidi and Yeo, 2020).

Figures 5.1 and 5.2 display the ABTS and DPPH radical scavenging activity of protein hydrolysates and those of protein isolates; the hydrolysates showed significantly higher values ($p<0.05$) when compared to their corresponding isolates. The significant difference between the unhydrolyzed and hydrolyzed samples implies that SPI antioxidant peptides produced during enzyme digestion can donate electron to decrease $ABTS^{\bullet+}$. The ABTS radical scavenging activity of hydrolysates ranged between 26 and 46%, while DPPH radical scavenging activity was from 17 to 36%. Sophia protein isolate (SPI) hydrolyzed with Alcalase exhibited the highest ($p < 0.05$) ABTS and DPPH radical scavenging ability,

whereas Flavourzyme hydrolyzed SPI showed the lowest radical scavenging activity. According to the previous study, the ability of food proteins and their hydrolysates to scavenge free radicals is influenced by a variety of factors, such as their size and the amino acid composition and sequence of the peptides, as well as the specificity of the protease (Ngo and Shahidi, 2021b; Girgih et al., 2011). For example, aromatic amino acid residues (Tyr, His, Trp, and Phe) have the ability to donate hydrogen atoms to electron-deficient radicals through a procedure known resonance stabilisation. This enhances the radical-scavenging properties of the amino acid residues. Alkaline proteases, such as Alcalase and Flavourzyme, produce hydrolysates with more antioxidant activity compared to enzymes such as Neutrase, papain, bromelain, and pepsin (He et al., 2013; Hu et al., 2020). The trends observed for the ABTS and DPPH radical scavenging activities of the sophia protein hydrolysates, generated upon treatment with Alcalase are comparable to those observed for camelina protein hydrolysates that were previously reported (Ngo and Shahidi, 2021b). Therefore, the previously reported investigation on the radical scavenging capabilities of hydrolysates from a variety of plant proteins is supported by our findings (He et al., 2013; Alashi et al., 2014; Ngo and Shahidi, 2021b).

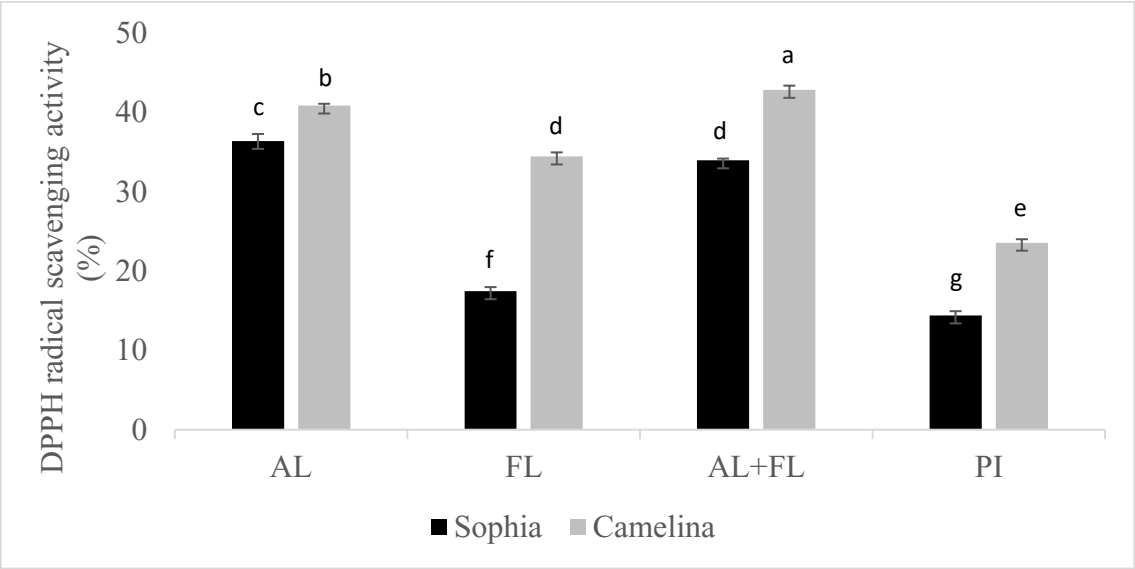


Figure 5.1 DPPH radical scavenging of sophia and camelina protein isolates (PI), and their hydrolysates prepared using enzymatic treatment (AL, Alcalase; FL, Flavourzyme; and AL+FL, Mixture of Alcalase and Flavourzyme)

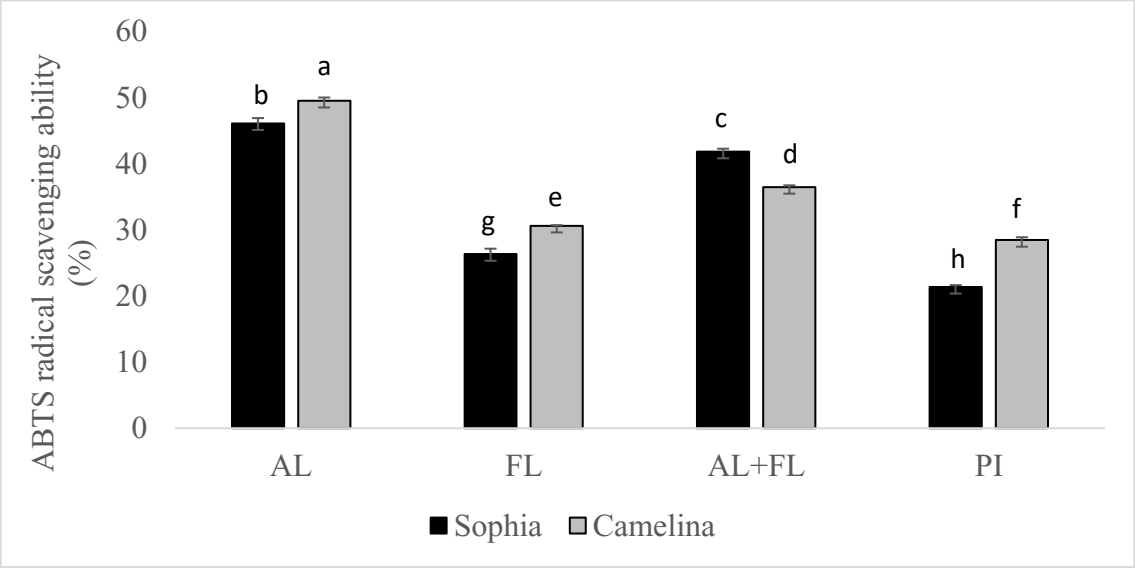


Figure 5.2 ABTS radical scavenging of sophia and camelina protein isolates (PI), and their hydrolysates prepared using enzymatic treatment (AL, Alcalase; FL, Flavourzyme; and AL+FL, Mixture of Alcalase and Flavourzyme)

All data represent means of triplicate determinations. Values followed by the same alphabets are not significantly different ($p > 0.05$) by Tukey's HSD test.

5.4.2 Hydroxyl radical scavenging activity and oxygen radical absorbance capacity (ORAC)

The most damaging forms of oxidative stress are caused by hydroxyl radicals, which interact with biomolecules such amino acids, proteins, DNA, and membrane lipids to cause severe cell damage (Ngo and Shahidi, 2021b). Moreover, oxygen radicals play a major role in oxidative stress, which is caused by an imbalance between antioxidants and reactive oxygen species (ROS) and leads to fatal diseases such as cancer, cardiovascular diseases, arthritis, diabetes, and aging (Ambigaipalan et al., 2015). In the present study, the hydroxyl radical scavenging and oxygen radical absorbance of camelina and sophia protein hydrolysates is showed in Table 5.1**.** The ability to scavenge hydroxyl radicals was highest in sophia and camelina protein hydrolysates prepared with Alcalase around 3.13 and 3.21 (µm of Histidine/mg protein), respectively, and lowest in those prepared with Flavourzyme at 1.91 and 2.5 (µm of Histidine/mg protein). There was a range of values for the oxygen radical absorption capacity of camelina/sophia protein hydrolysates, from 0.51 to 1.13 (µm of Trolox/mg protein). The results indicated that the ability to scavenge hydroxyl radicals and oxygen radical absorbance capacity of camelina hydrolysates are higher than that of sophia hydrolysates. According to the findings of the research, hydrolysates that were generated from alcalase gave the greatest value of hydroxyl radical scavenging ability as well as oxygen radical absorbance capacity. A significant difference ($p < 0.05$) existed in hydroxyl radical scavenging ability and oxygen radical absorbance capacity of sophia protein hydrolysates which decreased in the order AL > AL+FL > FL. Similar results for Alcalase-

assisted hydrolyzed products of date seed meals and camelina meals have been reported in the previous studies (Ambigaipalan et al., 2015; Ngo and Shahidi, 2021b)

Table 5.1 Hydroxyl radical scavenging activity and oxygen radical absorbance capacity (ORAC) of camelina/sophia protein hydrolysates

Sample	Protein hydrolysates	Hydroxyl radical scavenging activity (μm of Histidine/mg protein)	Oxygen radical absorbance capacity (μm of Trolox/mg protein)
Camelina	Alcalase	3.21 ± 0.03a	1.13 ± 0.05a
	Flavourzyme	2.50 ± 0.05b	0.62 ± 0.04b
	Alcalase + Flavourzyme	2.71 ± 0.16c	0.93 ± 0.03c
Sophia	Alcalase	3.13 ± 0.08a	1.03 ± 0.05a
	Flavourzyme	1.91 ± 0.05b	0.51 ± 0.01b
	Alcalase + Flavourzyme	2.63 ± 0.16c	0.93 ± 0.04c

Data presented as mean ± SD (n = 3). Results followed by the same lowercase letter in the column are not significantly different at $p < 0.05$.

5.4.3 Metal chelation activity

The Fenton reaction, involved lipid oxidation, leads to the degradation of hydroperoxide into volatile compounds. Chelation of transition metal ions (Fe, Cu, Co) by hydrolysates reduces the intensity of the pink Ferrozine-ferrous complex (Shahidi and Zhong, 2015). The chelation of metal ions by sophia protein hydrolysates revealed significantly better metal chelating activity than their respective protein isolates, as shown in Figure 5.3

and that of camelina hydrolysates are published (Ngo and Shahidi, 2021b). Furthermore, chelation of ferrous ions by sophia protein hydrolysates varied greatly in the order of AL > AL+ FL > FL. The highest metal chelation activity of sophia hydrolysates was observed in hydrolysates prepared by Alcalase, while AL+FL designed that of camelina hydrolysates (Ngo and Shahidi, 2021b). This capacity is correlated with the presence of amino acid residues such as histidine, cystine, tryptophan, aspartate, and glutamate on the surface of proteins and polypeptides, which have been found to bind divalent metal ions. Additionally, because of the imidazole group that it possesses, the presence of histidine at the N terminal can be connected to a considerable metal ion chelation (Ambigaipalan et al., 2015; Udenigwe et al., 2016). The findings suggest that peptides can form complexes with transition metal ions, thus retarding the oxidation process.

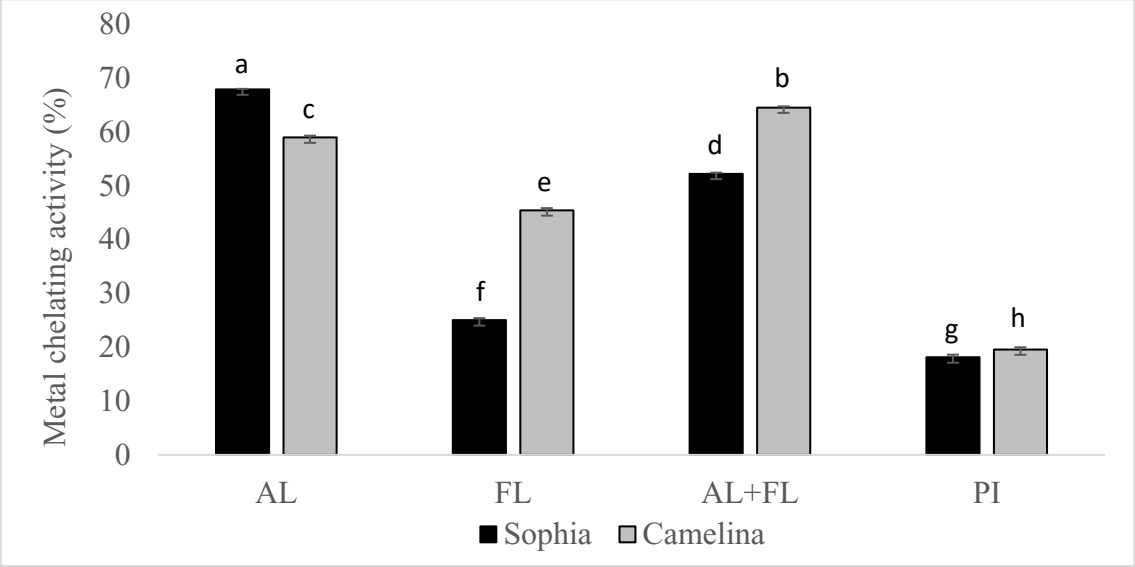


Figure 5.3 Metal chelation activity of sophia and camelina protein isolates (PI), and their hydrolysates prepared using enzymatic treatment (AL, Alcalase; FL, Flavourzyme; and AL+FL, Mixture of Alcalase and Flavourzyme)

All data represent means of triplicate determinations. Values followed by the same alphabets are not significantly different ($p > 0.05$) by Tukey's HSD test.

5.4.4 Inhibition against copper-induced LDL-cholesterol oxidation

LDL cholesterol is a key factor in transporting triacylglycerols and cholesterol to body cells. Plasma low-density lipoprotein (LDL) oxidation, caused by metal ions or reactive oxygen species, is considered one of the major risk factors for the development of atherosclerosis and coronary heart disease. Cupric ion has been shown to be effective in initiating the oxidation of EDTA-free human LDL, and the presence of both cholesteryl linoleate and cholesteryl arachidonate within the LDL core provides a rich source of lipid peroxidation substrate. In this assay, Cu^{2+} is used to induce *in vitro* human LDL-cholesterol oxidation, and the outcomes of conjugated dienes are measured at 234 nm. According to the Table 5.2, camelina and sophia protein hydrolysates and carnosine exhibited higher after 12 h of incubation, being 39.04−79.97 and 78.59%, respectively. Flavourzyme displayed the lowest amount of conjugated dienes production in comparison to all other treatments for protein hydrolysates. Camelina hydrolysates prepared by Alcalase has demonstrated its highest efficacy in inhibiting cupric ion-mediated LDL oxidation (79.97%). Camelina and sophia protein hydrolysates were found to have an inhibitory effect on human LDL cholesterol oxidation, which could be linked to their ability to chelate copper ions and scavenge free radicals in LDL. In comparison to date seed protein, sophia and camelina protein hydrolysates were found to have a similar inhibitory impact on LDL oxidation (Ambigaipalan and Shahidi, 2015).

Table 5.2 Inhibition against human LDL cholesterol oxidation by sophia/camelina protein hydrolysates and carnosine

Protein hydrolysates	LDL oxidation inhibition (%)	
	Sophia	Camelina
Carnosine	78.59 ± 0.11a	
Alcalase	73.66 ± 1.19b	79.97 ± 0.29b
Flavourzyme	39.04 ± 0.28c	45.94 ± 0.44c
Alcalase + Flavourzyme	72.39 ± 0.59b	62.68 ± 0.87d

Data presented as mean ± SD (n = 3). Results followed by the same lowercase letter in the column are not significantly different at $p < 0.05$.

5.4.5 Inhibition of hydroxyl and peroxyl radical-induced supercoiled DNA strand scission

Free radicals such as hydroxyl and peroxyl radicals generated in living cells, possess a more significant reduction potential, and can react with biomolecules, damaging DNA at both the phosphate backbone and the nucleotide bases. This can lead to mutation, carcinogenesis, and other pathological processes. As a result, limiting DNA oxidation is critical for maintaining cell health. In this experiment, AAPH, a cationic water-soluble azo substance, was used as the source of alkoxyl and alkyl peroxyl radicals to induce single strand cleavage of supercoiled plasmid DNA and assess the related protective effect of hydrolysates on DNA oxidation. This was carried out to find out if hydrolysates have a protective effect against DNA oxidation. Carnosine was chosen as a positive control due to its natural antioxidant properties. The results are shown in Table 5.3**.** Sophia and camelina protein

hydrolysates were found to reduce hydroxyl radical-induced DNA scission by 19.85 to 53.01% and 28.25 to 59.52%, respectively. Additionally, these hydrolysates exhibited a decrease in peroxyl radical-induced DNA oxidation by 62.83 to 87.10% and 75.82 to 88.77%, respectively. In both hydroxyl radical- and peroxyl radical-induced oxidation, hydrolysates that were produced by Alcalase and the combination of Alcalase and Flavourzyme showed a more potent inhibitory effect than carnosine. Similar findings were found for date seed protein hydrolysates, where supercoiled DNA retention was 13-33 and 47-83% for hydroxyl and peroxyl radical effects, respectively (Ambigaipalan and Shahidi, 2015). As a result, sophia and camelina protein hydrolysates have the potential to act as an appropriate dietary source for the protection of DNA against oxidation.

Table 5.3 Inhibition of hydroxyl and peroxyl radical-induced supercoiled DNA strand scission by sophia/camelina protein hydrolysates

Samples	Protein hydrolysates	DNA scission inhibition (%)	
		Hydroxyl radical	Peroxyl radical
Sophia	Carnosine	30.44 ± 1.36d	22.63 ± 1.15d
	Alcalase	53.01 ± 0.50a	87.10 ± 2.35a
	Flavourzyme	19.85 ± 0.87b	62.83 ± 1.37b
	Alcalase + Flavourzyme	46.35 ± 0.39c	86.52 ± 0.32a
Camelina	Alcalase	59.62 ± 2.21a	88.77 ± 0.75a
	Flavourzyme	28.25 ± 0.73b	75.82 ± 1.55b
	Alcalase + Flavourzyme	47.02 ± 0.35c	80.07 ± 1.36c

Data presented as mean ± SD (n = 3). Results followed by the same lowercase letter in the column are not significantly different at $p < 0.05$.

5.4.6 Amino acid composition

The amino acid content of protein hydrolysates has a major impact on their antioxidant activity and plays an important part in the physiological benefits that these substances provide. Table 5.4 summarizes the amino acid composition of sophia protein hydrolysates prepared by Alcalase and these correspond with those of sophia seeds in a previous study (Mohamed and Mahrous, 2009). Furthermore, the amino acid profile of camelina and sophia protein hydrolysates almost meets the recommendations of the World Health Organization/Food and Agriculture Organization (WHO/FAO) for the amounts of most essential amino acids in food products. However, the levels of methionine and histidine in sophia hydrolysates are quite low compared to those recommended by WHO/FAO. Besides, the amounts of most amino acids in camelina hydrolysates are higher significantly than that of sophia hydrolysates. The amino acid composition of camelina and sophia protein hydrolysates was rich in leucine, tryptophan, arginine, glutamic and aspartic. The high amounts of leucine found in hydrolysates may promote scavenging of superoxide radicals. Furthermore, negatively charged amino acids such as Glu and Asp have strong antioxidant properties because they are rich sources of electrons that can be donated to free radicals, while tyrosine residue can act as a potent hydrogen donor, and histidine can be credited with strong radical scavenging activity due to the decomposition of its imidazole ring (Ngo and Shahidi, 2021b; Udenigwe et al., 2016). As a result, the amino acid profile has been proposed as a method for predicting the antioxidant activity of protein hydrolysates. According to the

findings of this study, camelina/sophia protein hydrolysates have the potential to serve as a valuable contributor of protein to food.

Table 5.4 Amino acid composition of sophia/camelina protein hydrolysates (Alcalase) (g/100g)

Amino acid	Sophia hydrolysates	Camelina hydrolyses (9)	WHO/FAO
Essential amino acid (EAA)			
Histidine (His)	0.9	2.1	1.6
Isoleucine (Ile)	1.4	2.9	1.3
Leucine (Leu)	2.2	4.9	1.9
Lysine (Lys)	1.4	2.3	1.6
Methionine (Met)	0.6	1.5	1.7
Phenylalanine (Phe)	1.5	3.2	
Threonine (Thr)	1.3	2.8	0.9
Tryptophan (Trp)	2.1	1.3	0.5
Valine (Val)	1.6	3.8	1.3
Non EAA (NEEA)			
Alanine (Ala)	1.1	2.8	
Arginine (Arg)	2.8	7.0	
Aspartic acid +Asparagine (Asp+Asn)	3.0	6.4	
Cystine (Cys)	0.8	1.4	
Glutamic acid + Glutamine (Glu + Gln)	5.8	12.7	
Glycine (Gly)	1.8	3.2	
Proline (Pro)	1.5	3.4	
Serine (Ser)	1.3	3.0	
Tyrosine (Tyr)	1.2	2.4	
Phenylalanine + Tyrosine (Phe + Tyr)	2.7	3.3	1.9

5.4.7 Peptide ranker analysis

As shown by the research results given above, the protein hydrolysate produced by Alcalase has high antioxidant activity. In this connection, peptideRanker (PepRank) is an *in silico* tool that has been used to predict the potential of bioactive peptides prepared using Alcalase. In general, any peptide with a threshold more than 0.5 is considered bioactive. Besides, the false optimistic predictions reduce from 11 and 16% at a 0.5 threshold to 2 and 6% at a 0.8 threshold for long and short peptides, respectively. PepRank is more dependable that the peptide is bioactive when the expected probability is close to 1 (Mooney et al., 2012). Following LC-MS/MS analysis, identified peptides from sophia/camelina protein hydrolysates were subjected to a peptide ranker tool to predict possible activity with a 0.8 threshold score. Table 5.5 shows 9 and 33 peptide sequences that were found to be potentially bioactive in sophia and camelina hydrolysates, respectively. These were with a threshold score of 0.8 to 0.96. FGFGPGL and GPPSGGGGGGGGGGGGGK peptide had the highest threshold score (0.96) while the lowest score was for SFPLPEL (0.8). Previous studies have shown that biological activity of peptide sequence is determined by amino acid position and composition (Garg et al., 2018; Pooja et al., 2017). Therefore, the BIOPEP database was employed to investigate the antioxidative, ACE inhibitory, and DPP IV inhibitory effect of expected peptides (Minkiewicz et al., 2019).

Table 5.5 Ranking of the predicted bioactive peptide sequences prepared sophia and camelina protein obtained by PepRank

Samples	PepRank	Peptide sequence
Sophia hydrolysates	0.96	FGFGPGL
	0.89	SSTSGPAFNAGRSIWLPGWL
	0.85	CAYDVAPGGLL
	0.85	SLCGIPPL
	0.83	PMITGFM
	0.82	FVPVTGLWM
	0.82	WYTICICIL
	0.82	RAPWLEPL
	0.81	LGMLPGL
Camelina hydrolysates	0.96	GPPSGGGGGGGGGGGGK
	0.94	IDLFFVFL
	0.94	AAMGGFPGGGGGAHALGVL
	0.92	LNPCFTGGPLM
	0.92	NGGGGGGGGGGGPPKMVL
	0.91	PPPPGAL
	0.9	GGGGGGGFGGGAGGGLGGGGGL
	0.9	GGSPGIGGGL
	0.89	DIPPPRGPL
	0.89	GGGIGGGGGGGGGFGGGSGSGGGAGGGAGGGL

0.89	LLGNGIGSGGGHGGKGGRVCY
0.88	FGGGNLPAFVL
0.88	AWPDKNPFFPSDPY
0.88	FVPPFNPY
0.86	DFSIFSPL
0.85	FGGGNIPAFVL
0.84	GGGGGGGGPPAMSM
0.84	GLDPPDLPM
0.83	VIGPGLGRDPFLL
0.83	GGGAGGGGGL
0.83	VFGSGLLGAFL
0.83	FNAPIYL
0.83	GPFGVIRPPL
0.82	IHPIPPL
0.82	AGAATGGFL
0.82	NSGGGGGGWKGGGGQGGGWKGGGGQ
0.81	QFQWIEFK
0.81	FHWDLPQ
0.81	FGGYAPGILSPSPAML
0.81	FSFSPTVFDMILK
0.81	FGWDKDL
0.8	SFPLPEL

5.4.8 *In silico* predictions of potential bioactive peptides from sophia/camelina protein hydrolysate

The tool "Profiles of potential biological activity" from the BIOPEP-UWM database was used to predict the bioactive potential of selected peptides. These peptides were screened to investigate the type and the locations of the bioactive fragments in peptide sequences. According to BIOPEP-UWM database, Tables 5.6 indicate profiles of potential biological activity of camelina/sophia protein hydrolysates, including antioxidative, ACE- and DPP IV-inhibitory activities. Five of the 9 peptide sequences, and 12 of the 33 peptides sequences released from sophia and camelina hydrolysates, respectively were shown to have the potential to be antioxidative, and all selected sophia/camelina-derived bioactive peptides were found to have the ability to inhibit ACE and DPP-IV activities. All active fragments are dipeptides or tripeptides except for GFGPGL which are easily absorbed in the gastrointestinal tract. The total number of ACE-inhibitory and DPP IV-inhibitory dipeptides is significantly higher than the total of tripeptides.

Interestingly, tryptophan (W) was the most common amino acid residue present in antioxidative peptides derived from sophia hydrolysates, such as FVPVTGLWM, WYTICICIL, and RAPWLEPL. Besides, GPP was the most common bioactive sequence identified in peptides derived from camelina hydrolysates. Furthermore, presence of hydrophobic amino acids including alanine (A), valine (V), leucine (L), isoleucine (I), proline (P), phenylalanine (F), tyrosine (Y), methionine (M), histidine (H), and tryptophan (W) is responsible for the antioxidant activity. For instance, tyrosine and phenylalanine residues can act as potent hydrogen donors, and histidine can be credited with strong radical scavenging

activity due to its imidazole ring (Samtiya et al., 2021; Ngo and Shahidi, 2021b). Previous research has shown that several peptides found in sophia/camelina protein hydrolysates are potent antioxidants, and these peptides come from a variety of animal and plant sources. According to the database, AY, PW, LW and WY, IY and IR were identified from okara protein, buckwheat, marine bivalve and bovine beta-lactoglobulin, jack bean and canavalin protein, respectively.

The highest number of potential ACE inhibitory dipeptides and tripeptides (14) were found in the sequence SSTSGPAFNAGRSIWLPGWL. Several peptides included in sophia/camelina protein hydrolysates have previously been identified as potent ACE inhibitors from various animal and plant sources. For example, GP, PL, GPL, LPG were identified from Alaskan pollack skin, while CF, SF, GPP, FVP were also previously confirmed from shark meat hydrolysate, garlic, wheat gliadin, and soya milk, respectively. Previous research has demonstrated that characteristics of peptides, such as chain length, composition, and sequence, can significantly affect their ACE-inhibitory activities. For example, presence of hydrophobic amino acids such proline (P), phenylalanine (F), isoleucine (I), valine (V), methionine (M), leucine (L), and alanine (A) close to the C-terminus of peptides promotes their binding to ACE and indeed inhibit the enzyme more effectively (Ambigaipalan et al., 2015; Daskaya-Dikmen et al., 2017). Additionally, the effect of proline on ACE inhibitory activity is associated with its imidazole ring, which interacts strongly with the amino acid residues at the active centers of ACE. Proline was present in most ACE inhibitory peptide sequences. Thus, peptides derived from sophia/camelina meals are effective in inhibiting ACE. Alcalase was the most promising

enzyme used for the isolation of both antioxidant and antihypertensive peptides, consistent with previous research on bioactive peptides derived from edible seeds (Samtiya et al., 2021).

According to Table 5.6, all the active segments are dipeptides or tripeptides, with dipeptides being most abundant and effective DDP IV inhibitors. Besides, in active dipeptides and tripeptides, proline and alanine were the two most common amino acids such as GP, GA, PG, PA, AG, IP, LP, AP, PP, VP, APG, GPA, PPL. This corresponds to some previous research results that peptides having proline or alanine residues in their sequences have potential DPP-IV inhibitory activity (Cermeno et al., 2019; Ding et al., 2022). According to the findings, it has been demonstrated that multifunctional peptides exhibit more than one major physiologically relevant bioactive characteristic. Because of this, there is more interest in multifunctional peptides now found in food proteins. Peptides, on the other hand, are susceptible to degradation during digestion in the gastrointestinal tract, and the biological activity of peptides can be either activated or inactivated depending on the structure and function of the peptide. Since *in silico* digestion is an appropriate method for predicting the release and resistance of bioactive peptides, the predicted peptides that were derived from the sophia/camelina protein hydrolysates were put through this process.

Table 5.6 *In silico* predictions of potential bioactive peptides from sophia and camelina protein hydrolysate

Sample	Peptide sequence	Bioactive segment sequence		
		Antioxidative peptides	ACE-inhibitory peptides	DPP IV inhibitory peptides
Sophia hydrolysates	FGFGPGL	GFGPGL	PGL, GP, GF, GL, FG, PG	GP, GL, GF, PG
	SSTSGPAFNAGRSIWLPGWL	-	GPA, LPG, P, IW, GW, AF, AG, GR, SG, PG, WL, SGP, ST, LP	GP, PA, LP, GPA, WL, AF, AG, FN, GW, IW, NA, PG, SI, TS
	CAYDVAPGGLL	AY	VAP, AY, AP, GL, GG, PG	VA, AP, LL, APG, GL, AY, GG, PG, YD
	SLCGIPPL	-	IPP, PL, IP, GI, PP, PPL	PP, IP, SL, PL, PPL, GI
	PMITGFM	-	GF, TG	GF, MI, PM, TG
	FVPVTGLWM	LWM, LW	LW, VP, GL, TG	VP, GL, WM, LW, PV, TG, VT
	WYTICICIL	WY, WYT	FVP, WM	WY, YT, IL, TI
	RAPWLEPL	PWL, PW	PL, AP, RA, WL	AP, RA, WP, PL, WL, PW
	LGMLPGL	-	PGL, LPG, GM, GL, LG, PG, LP	LP, GL, ML, PG

Camelina hydrolysates	GPPSGGGGGGGGGGGGK	GPP	GP, GK, GG, SG, GPP, PP	GP, PP, GG, PS
	IDLFFVFL	-	VF, LF, FF	FL, VF, FF
	AAMGGFPGGGGGAHALGVL	AH, GAH	FP, AA, GF, GA, GV, MG, GG, LG, PG, AH, LGV	HA, FP, GA, AL, AA, AH, GF, GG, GV, MG, PG
	LNPCFTGGPLM	-	LNP, GPL, GP, PL, GG, TG, CF, LN	GP, NP, PL, GG, LM, LN, TG
	NGGGGGGGGGGGPPKMVL	GPP	GP, GG, NG, PPK, GPP, PP	GP, PP, GG, MV, NG, PK, VL
	PPPPGAL	-	GA, PG, PP, PPP	PPPP, PP, GA, AL, PPG, PG
	GGGGGGGFGGGAGGGLGGGGGL	-	GF, GA, GL, AG, FG, GG, LG, FGG	GA, GL, AG, GF, GG
	GGSPGIGGGL	-	IG, GI, GL, GS, GG, PG	SP, GL, GG, GI, PG
	DIPPPRGPL	-	IPP, PR, GPL, GP, PL, IP, PP, PPP, RG	GP, PP, IP, PL, RG
	GGGIGGGGGGGGGFGGGSGS-GGGAGGGAGGGL	-	GF, IG, GA, GL, AG, FG, GS, GG, SG, FGG	GA, GL, AG, GF, GG, GI
	LLGNGIGSGGGHGGKGGRVCY	-	IG, GI, GH, GR, KG, GS, GK, HG, GG, SG, LG, NG, GHG	LL, GG, GH, GI, KG, NG
	FGGGNLPAFVL	-	AF, FG, GG, FGG, LP	PA, LP, AF, GG, NL, VL

AWPDKNPFFPSDPY	AW	FP, AW, FF	WP, FP, NP, AW, DP, PF, PS, PY, FF
FVPPFNPY	-	VPP, VP, PP, FVP	PP, VP, NP, FN, PF, PY
DFSIFSPL	-	PL, IF, DF	SP, PL, SI
FGGGNIPAFVL	-	IPA, IP, AF, FG, GG, FGG	PA, IPA, IP, AF, GG, VL
GGGGGGGGPPAMSM	GPP	GP, GG, GPP, PP	GP, PP, PA, GG
GLDPPDLPM	LPM	DLP, GL, PP, LP	PP, LP, GL, DP, PM
VIGPGLGRDPFLL	-	PGL, GP, IG, GL, GR, LG, PG	GP, LL, FL, GL, DP, PF, PG, VI
VFGSGLLGAFL	-	VF, AF, GA, GL, FG, GS, SG, LG, AFL	GA, GL, AG, GG
FNAPIYL	IY	IY, YL, AP	AP, FN, NA, PI, YL
GPFGVIRPPL	IR	GP, PL, IRP, RP, FG, GV, PP, RPP	GP, PP, RP, PL, PPL, GV, IR, PF, VI
IHPIPPL	-	IPP, PL, IP, PP, HP, PPL	PP, IP, HP, PL, PPL, IH, PI
AGAATGGFL	GAA	AA, GF, GA, AG, GG, TG	GA, FL, AA, AG, AT, GF, GG, TG
NSGGGGGGWKGGGGQGG -GWKGGGGQ	-	GW, KG, GQ, GG, QG, SG	WK, GG, GW, KG, QG
QFQWIEFK	-	IE, FQ, EF	WI, FQ, QF, QW

FHWDLPQ	-	DLP, PQ, LP	LP, HW, PQ, WD, LPQ
FGGYAPGILSPSPAML	-	GGY, GY, LSP, AP, YA, GI, FG, GG, PG, IL, FGG	AP, PA, APG, SP, GG, GI, GY, IL, ML, PG, PS, YA
FSFSPTVFDMILK	LK	VF, SF, PT, DM, IP	SP, IL, MI, PT, SF, TV, VF
FGWDKDL	KD	GW, FG	GW, WD
SFPLPEL	EL, PEL	PLP, FP, PL, SF, LP	LP, FP, PL, SF
SGLGGGQGIGGGSGTGM	-	IG, GI, GM, GL, GS, GQ, GT, GG, QG, SG, LG, TG, GTG	GL, GG, GI, QG, TG

5.4.9 *In silico* simulated gastrointestinal (GI) digestion of sophia/camelina protein hydrolysates

Peptides that make up the building blocks of the human body are created when protein products are digested. To determine whether peptides can provide a health benefit, they must be subjected to simulated digestive conditions (Udenigwe and Aluko, 2012; Wu et al., 2006). In this perspective, simulating GI digestion *in silico* is a valuable tool for determining the bioactive potential of sophia/camelina peptide before assessing its bioavailability and bio accessibility *in vivo*. The sequences of protein hydrolysates were subjected to *in sili*co proteolysis using pepsin (EC 3.4.23.1), trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) for the prediction of theoretically released peptides by the enzymatic action program available in BIOPEP tool. In the present study, *in silico* proteolysis demonstrates the ability of given enzymes to release the antioxidative and the ACE-inhibitory peptides from sophia/camelina protein hydrolysates.

After simulated digestion process of sophia hydrolysates, none of the peptides displayed antioxidative potential, as predicted. This could be because there is insufficient information in the antioxidative peptide database, or it could be because gastrointestinal condition cleaves certain peptides (Udenigwe, 2014). The results showed that 3 and 3 out of the 9 peptides identified from sophia hydrolysates were anticipated to release potent ACE inhibitory and DPP IV inhibitory fragments from their original sequences, respectively (Table 5.7). It is interesting to note that FGFGPGL possesses powerful ACE inhibitory and DPP IV inhibitory properties. GF is an active fragment sequence that can inhibit ACE as well as DPP IV. According to the findings given in Table 5.7, the bioactive the bioactive potential

upon GI digestion of peptides derived from camelina protein hydrolysate was reported. The results show that two peptides (AWPDKNPFFPSDPY, SGLGGGQGIGGGSGTGM and SFPLPEL) have the potential antioxidant after the simulated digestion process. There are 13 out of the 33 peptides identified from camelina hydrolysates were predicted to release potent ACE inhibitory fragments from their original sequences. The ACE-inhibitory dipeptides are the most abundantly released from camelina protein hydrolysates, whereas few tri-peptides sequences were found. Importantly, the frequency of release of potent ACE inhibitor peptides from each identified peptide is different because some peptides have more than one active fragment embedded in their sequence. For instance, SFPLPEL sequence was predicted to release three active ACE inhibitory sequences, namely PL, SF and PEL.

The "enzyme(s) action" option of the BIOPEP database is used to determine the degree of hydrolysis (DHt), the release frequency (A_E), and the value of relative release frequency (W). A_E and W are the two most important parameters to consider when estimating the potential release of bioactive peptides from protein sequences (Pooja et al., 2017). *In silico* digestion revealed sophia and camelina hydrolysates as having degrees of hydrolysis ranging from 12.50 (DIPPPRGPL) to 66.67% (FHWDLPQ), as indicated in Table 5.8. The highest A_E value (0.29) was observed in peptide SFPLPEL, whereas the lowest (0.06) was FGGYAPGILSPSPAML. Regarding camelina hydrolysates, the highest relative frequency of the release of fragments with a given activity by selected enzymes (W) was 1.0 in AWPDKNPFFPSDPY, whereas AAMGGFPGGGGGAHALG (0.06) is the lowest value. After *in silic*o simulated digestion of camelina meals derived peptides, most of the bioactive fragments possess proline (P), glycine (G), leucine (L) phenylalanine (F) in their dipeptide

and tripeptide sequences. The bioactive motifs that result contain VF, GPL, AW, DF, GL, GR, PPL, PQ, GGY, SF, DM, IL, GW, PL, PEL. Previous studies using *in silico* methods to identify biopeptides in proteins from a variety of plants and animals showed up similar results. According to the findings of several studies, the presence of hydrophobic amino acids (such as leucine, serine, and methionine) or aromatic amino acids (such as phenylalanine) in these peptide can boost the inhibition of ACE and DPP IV (Wu et al., 2006; Zhu et al., 2017). In addition, peptides that contain proline in their sequences have the potential to be highly effective ACE and DPP-IV inhibitors (Cermeno et al., 2019). Multiple bioactivities can boost the impact of these peptides on more than one disease target or symptom since many human diseases are connected in origin and progression. Hence, sophia/camelina protein hydrolysates may be used in nutraceutical or functional food products, according to these discoveries.

Table 5.7 Remaining bioactive properties after *in silico* simulated gastrointestinal digestion of peptides prepared sophia and camelina protein hydrolysate.

Sample	Peptide	Results of enzyme action	Location of released peptides	Active fragment sequence	Location	Bioactivity of identified peptide
Sophia hydrolysates	FGFGPGL	F - GF - GPGL	[1-1],[2-3],[4-7]	GF	[2-3]	ACE inhibitor, DPP IV inhibitor
	SSTSGPAFNAGRSIWLPGWL	SSTSGPAF - N - AGR-SIW - L - PGW - L	[1-8],[9-9],[10-12],[13-15], [16-16],[17-19],[20-20]	-	-	-
	CAYDVAPGGLL	CAY - DVAPGGL - L	[1-3],[4-10],[11-11]	-	-	-
	SLCGIPPL	SL - CGIPPL	[1-2],[3-8]	SL	[1-2]	DPP IV inhibitor
	PMITGFM	PM - ITGF - M	[1-2],[3-6],[7-7]	PM	[1-2]	DPP IV inhibitor
	FVPVTGLWM	F - VPVTGL - W - M	[1-1],[2-7],[8-8],[9-9]	-	-	-
	WYTICICIL	W - Y - TICICIL	[1-1],[2-2],[3-9]	-	-	-
	RAPWLEPL	R - APW - L - EPL	[1-1],[2-4],[5-5],[6-8]	-	-	-
	LGMLPGL	L - GM - L - PGL	[1-1],[2-3],[4-4],[5-7]	PGL GM	[5-7] [2-3]	ACE inhibitor ACE inhibitor
Camelina hydrolysates	GPPSGGGGGGGGGGGGK	-	-	-	-	-
	IDLFFVFL	IDL - F - F - VF - L	[1-3],[4-4],[5-5], [6-7],[8-8]	VF	[6-7]	ACE inhibitor; DPP IV inhibitor
	AAMGGFPGGGGGAHALGVL	AAM - GGF - PGGGGGAH - AL - GVL	[1-3],[4-6],[7-14], [15-16],[17-19]	AL	[15-16]	DPP IV inhibitor

LNPCFTGGPLM	L - N - PCF - TGGPL - M	[1-1],[2-2],[3-5], [6-10],[11-11]	-	-	-
NGGGGGGGGGGGPPKMVL	N - GGGGGGGGGGGPPK -M - VL	[1-1],[2-15], [16-16],[17-18]	VL	[17-18]	DPP IV inhibitor
PPPPGAL	-	-	-	-	-
GGGGGGGFGGGAGG-GLGGGGGL	GGGGGGGF - GGGAGGGL - GGGGGL	[1-8],[9-16],[17-22]	-	-	-
GGSPGIGGGL	-	-	-	-	-
DIPPPRGPL	DIPPPR - GPL	[1-6],[7-9]	GPL	[7-9]	ACE inhibitor
GGGIGGGGGGGGGFGGGSG-SGGGAGGGAGGGL	GGGIGGGGGGGGGF - GGGSGSGGGAGGGAGGGL	[1-14],[15-32]	-	-	-
LLGNGIGSGGGHGGKGGRVCY	L - L - GN - GIGSGGGH -GGK - GGR - VCY	[1-1],[2-2],[3-4],[5-12],	-	-	-
FGGGNLPAFVL	F - GGGN - L - PAF - VL	[1-1],[2-5],[6-6], [7-9],[10-11]	VL	[10-11]	DPP IV inhibitor
AWPDKNPFFPSDPY	AW - PDK - N - PF - F - PSDPY	[1-2],[3-5],[6-6], [7-8],[9-9],[10-14]	AW	[1-2]	ACE inhibitor; DPP IV inhibitor; antioxidative
			PF	[7-8]	DPP IV inhibitor
FVPPFNPY	F - VPPF - N - PY	[1-1],[2-5],[6-6],[7-8]	PY	[7-8]	DPP IV inhibitor
DFSIFSPL	DF - SIF - SPL	[1-2],[3-5],[6-8]	DF	[1-2]	ACE inhibitor
FGGGNIPAFVL	F - GGGN - IPAF - VL	[1-1],[2-5],[6-9],[10-11]	VL	[10-11]	DPP IV inhibitor
GGGGGGGGPPAMSM	GGGGGGGGPPAM - SM	[1-12],[13-14]	-	-	-
GLDPPDLPM	GL - DPPDL - PM	[1-2],[3-7],[8-9]	GL	[1-2]	ACE inhibitor; DPP IV inhibitor
			PM	[8-9]	DPP IV inhibitor
VIGPGLGRDPFLL	VIGPGL - GR - DPF - L - L	[1-6],[7-8],[9-11], [12-12],[13-13]	GR	[7-8]	ACE inhibitor
GGGAGGGGGL	-	-	-	-	-

VFGSGLLGAFL	VF - GSGL - L - GAF - L	[1-2],[3-6],[7-7], [8-10],[11-11]	VF	[1-2]	ACE inhibitor; DPP IV inhibitor
FNAPIYL	F - N - APIY - L	1-1],[2-2],[3-6],[7-7]	-	-	-
GPFGVIRPPL	GPF - GVIR - PPL	[1-3],[4-7],[8-10]	PPL	[8-10]	ACE inhibitor; DPP IV inhibitor
IHPIPPL	IH - PIPPL	[1-2],[3-7]	IH	[1-2]	DPP IV inhibitor
AGAATGGFL	AGAATGGF - L	[1-8],[9-9]	-	-	-
NSGGGGGGWKGGGGQGG-GWKGGGGQ	N- SGGGGGGW - K - GGGGQGGGW - K - GGGGQ	[1-1],[2-9],[10-10], [11-19],[20-20],[21-25]	-	-	-
QFQWIEFK	QF - QW - IEF - K	[1-2],[3-4],[5-7],[8-8]	QF	[1-2]	DPP IV inhibitor
			QW	[3-4]	DPP IV inhibitor
FHWDLPQ	F - H - W - DL - PQ	[1-1],[2-2],[3-3], [4-5],[6-7]	PQ	[6-7]	ACE inhibitor; DPP IV inhibitor
FGGYAPGILSPSPAML	F - GGY - APGIL - SPSPAM - L	[1-1],[2-4],[5-9], [10-15],[16-16]	GGY	[2-4]	ACE inhibitor
FSFSPTVFDMILK	F - SF - SPTVF - DM - IL - K	1-1],[2-3],[4-8], [9-10],[11-12],[13-13]	SF	[2-3]	ACE inhibitor; DPP IV inhibitor
			DM	[9-10]	ACE inhibitor
			IL	[11-12]	ACE inhibitor; DPP IV inhibitor
FGWDKDL	F - GW - DK - DL	[1-1],[2-3],[4-5],[6-7]	GW	[2-3]	ACE inhibitor; DPP IV inhibitor
SFPLPEL	SF - PL - PEL	[1-2],[3-4],[5-7]	SF	[1-2]	ACE inhibitor; DPP IV inhibitor
			PL	[3-4]	ACE inhibitor; DPP IV inhibitor
			PEL	[5-7]	antioxidative

Table 5.8 *In silico* proteolysis of sophia and camelina protein hydrolysate for release bioactive peptides sequences

Sample	Peptide	Active fragment sequence	DHt (%)	A_E	W	Activity
Sophia hydrolysates	FGFGPGL	GF	33.33	0.14	0.14	ACE inhibitor
				0.14	0.25	DPP IV inhibitor
	SLCGIPPL	SL	14.29	0.13	0.17	DPP IV inhibitor
	PMITGFM	PM	33.33	0.14	0.25	DPP IV inhibitor
	LGMLPGL	PGL, GM	50	0.29	0.29	ACE inhibitor
Camelina hydrolysates	IDLFFVFL	VF	57.14	0.13	0.33	ACE inhibitor
				0.13	0.33	DPP IV inhibitor
	AAMGGFPGGGGGAHALGVL	AL	22.22	0.05	0.06	DPP IV inhibitor
	NGGGGGGGGGGGPPKMVL	VL	17.65	0.06	0.06	DPP IV inhibitor
	DIPPPRGPL	GPL	12.5	0.11	0.1	ACE inhibitor
	FGGGNLPAFVL	VL	40	0.09	0.14	DPP IV inhibitor
	AWPDKNPFFPSDPY	AW, PF	38.46	0.07	0.33	ACE inhibitor
				0.14	0.22	DPP IV inhibitor
				0.07	1	Antioxidative
	FVPPFNPY	PY	42.85	0.13	0.17	DPP IV inhibitor
	DFSIFSPL	DF	28.57	0.13	0.33	ACE inhibitor
	FGGGNIPAFVL	VL	30	0.09	0.14	DPP IV inhibitor
	GLDPPDLPM	GL, PM	25	0.11	0.25	ACE inhibitor
				0.22	0.4	DPP IV inhibitor

VIGPGLGRDPFLL	GR	33.33	0.08	0.14	ACE inhibitor
VFGSGLLGAFL	VF	40	0.09	0.11	ACE inhibitor
			0.09	0.17	DPP IV inhibitor
GPFGVIRPPL	PPL	22.22	0.1	0.1	ACE inhibitor
			0.1	0.11	DPP IV inhibitor
IHPIPPL	IH	16.67	0.14	0.14	DPP IV inhibitor
QFQWIEFK	QF, QW	42.86	0.25	0.5	DPP IV inhibitor
FHWDLPQ	PQ	66.67	0.14	0.33	ACE inhibitor
			0.14	0.2	DPP IV inhibitor
FGGYAPGILSPSPAML	GGY	26.67	0.06	0.09	ACE inhibitor
FSFSPTVFDMILK	SF, DM, IL	41.67	0.23	0.6	ACE inhibitor
			0.15	0.29	DPP IV inhibitor
FGWDKDL	GW	50	0.14	0.5	ACE inhibitor
			0.14	0.5	DPP IV inhibitor
SFPLPEL	PL, SF, PEL	33.33	0.29	0.4	ACE inhibitor
			0.29	0.5	DPP IV inhibitor
			0.14	0.5	Antioxidative

5.4.10 Toxicity and allergenicity prediction of sophia-derived bioactive peptide fractions after *in silico* digestion

The toxicity of bioactive peptides is a major concern when developing peptide-based nutraceuticals or functional food ingredients. The ability to predict the toxicity of therapeutic peptides prior to their synthesis is critical for reducing the time and money spent developing peptide-based drugs (Ji et al., 2019). Besides, the majority of allergens are proteins present in a variety of plant and animal sources, resulting in a global problem that is continuously deteriorating (Pooja et al., 2017). As a result, all peptides derived from sophia protein hydrolysates following *in silico* digestion must be assessed for potential toxicity and allergenicity using the ToxinPred and allergen FP v.1.0 tools, respectively (Gupta et al., 2013; Pooja et al., 2017; Gupta et al., 2015). Table 5.9 illustrates that the low molecular weight dipeptides (GF, SL, GM, VF, AL, VL, AW, PF, PY, DF, GL, PM, GR, IH, QF, QW, PQ, SF, DM, IL, GW, PL) and tripeptides (PGL, GPL, PPL, GGY, PEL) that are released from sophia and camelina protein hydrolysates *in silico* are non-toxin. Non-toxic peptides are primarily composed of the amino acids such as Val, Thr, Arg, Gln, Met, Leu, Lys, Ile, Phe, and Ala. These findings are comparable to those obtained with peptides derived from other protein sources, such as tubers and quinoa, rice bran, and flaxseed protein, all of which were found to be non-toxic (Mudgil et al., 2020; Pooja et al., 2017; Ji et al., 2019; Ibrahim et al., 2018).

According to Table 5.9, 4 out of 5 sophia-derived bioactive peptide fractions were classified as probable non-allergens, while 16 of 23 camelina-derived bioactive peptide fractions were classified as probable allergens and 6 peptides were probable non-allergens.

Moreover, only VF is considered as non-allergen. The isoelectric points (pI) of the predicted bioactive peptides were found to be in the pH range of 3.80–10.11. Besides, 18 peptides have an isoelectric point of 5.88 with 0 net charge. The dipeptides (DF, DM) and tripeptides (PEL) showed negative charge (-1) with a pI of 3.8 and 4.0, respectively, whereas GR had positive charge (+1) with a pI of 10.11. Thus, bioactive peptides with an alkali isoelectric point had strong water solubility, whereas peptides with an acidic isoelectric point had poor water solubility. Some authors have investigated charge, peptide sequence, low molecular weight, hydrophobicity, and hydrophilicity as important structural properties for food-derived peptide bioavailability (Peredo-Lovillo et al., 2022; Sun et al., 2020). In the present study, the active fragment fraction had molecular weights ranging from 188.25 Da (GL) to 357.44 Da (PEL). The hydrophobicity of the bioactive peptides was shown to be in the range of -0.38 (PQ) to 0.57 (VF), whereas their hydrophilicity varied from -2 (VF) to 0.85 (DM). As the results, the peptides under consideration have the potential to be utilised in the food industry as functional ingredients.

Table 5.9 Prediction of toxicity and allergenicity of the potential bioactive fragments of sophia and camelina protein hydrolysate obtaining after the simulation *in silico* digestion

Sample	Active fragment sequence	Hydrophobicity	Hydrophilicity	Charge	pI	Molecular weight (Da)	Toxin Prediction	Allergenicity Prediction
Sophia hydrolysates	GF	0.39	-1.25	0	5.88	222.26	Non-Toxin	Probable allergen
	SL	0.14	-0.75	0	5.88	218.27	Non-Toxin	Probable non-allergen
	PM	0.1	-0.65	0	5.88	246.34	Non-Toxin	Probable non-allergen
	GM	0.21	-0.65	0	5.88	206.28	Non-Toxin	Probable non-allergen
	PGL	0.21	-0.6	0	5.88	285.38	Non-Toxin	Probable non-allergen
Camelina hydrolysates	VF	0.57	-2	0	5.88	264.34	Non-Toxin	Non-allergen
	AL	0.39	-1.15	0	5.88	202.27	Non-Toxin	Probable allergen
	VL	0.54	-1.65	0	5.88	230.33	Non-Toxin	Probable non-allergen
	GPL	0.21	-0.6	0	5.88	285.38	Non-Toxin	Probable allergen
	AW	0.31	-1.95	0	5.88	275.32	Non-Toxin	Probable allergen
	PF	0.27	-1.25	0	5.88	262.32	Non-Toxin	Probable allergen

PY	-0.03	-1.15	0	5.88	278.32	Non-Toxin	Probable allergen
DF	-0.05	0.25	-1	3.8	280.29	Non-Toxin	Probable allergen
GL	0.35	-0.9	0	5.88	188.25	Non-Toxin	Probable allergen
PM	0.1	-0.65	0	5.88	246.34	Non-Toxin	Probable non-allergen
GR	-0.8	1.5	1	10.11	231.27	Non-Toxin	Probable allergen
PPL	0.13	-0.6	0	5.88	325.44	Non-Toxin	Probable allergen
IH	0.16	-1.15	0.5	7.1	268.34	Non-Toxin	Probable allergen
QF	-0.04	-1.15	0	5.88	293.34	Non-Toxin	Probable allergen
QW	-0.16	-1.6	0	5.88	332.38	Non-Toxin	Probable allergen
PQ	-0.38	0.1	0	5.88	243.28	Non-Toxin	Probable allergen
GGY	0.11	-0.77	0	5.88	295.33	Non-Toxin	Probable non-allergen
SF	0.17	-1.1	0	5.88	252.28	Non-Toxin	Probable non-allergen
DM	-0.23	0.85	-1	3.8	264.31	Non-Toxin	Probable allergen
IL	0.63	-1.8	0	5.88	244.36	Non-Toxin	Probable non-allergen
GW	0.27	-1.7	0	5.88	261.3	Non-Toxin	Probable non-allergen
PL	0.23	-0.9	0	5.88	228.31	Non-Toxin	Probable allergen
PEL	-0.05	0.4	-1	4	357.44	Non-Toxin	Probable allergen

5.4.11 *In silico* evaluation of drug-likeness bioactive peptides derived from protein hydrolysates

In recent years, ADME computation, which stands for absorption, distribution, metabolism, and excretion, has been very helpful in the *in silico* prediction of the drug-likeness of bioactive peptides. SwissADME is a web-based tool for predicting and assessing small molecule pharmacokinetics, drug-likeness, and medicinal chemistry friendliness (Ji et al., 2020; Iwaniak et al., 2020). The assessment of drug-likeness, which determines the prospect of a component becoming an oral drug, is one of the SwissADME evaluation methods. Because of its flexibility, this method is considered a cost-effective alternative to time-consuming experimental methods (Daina et al., 2017; Daina et al., 2016). SwissADME was used to study physicochemical features such as the number of rotatable bonds (ROTB), hydrogen bond acceptors (HBA), hydrogen bond donors (HBD), topological polar surface area (TPSA), water solubility, lipophilicity, drug-likeness (Lipinski filter and bioavailability score), and pharmacokinetics (GI absorption). The drug-like characteristics of multifunctional dipeptides and tripeptides that were released from sophia and camelina hydrolysates were used to predict the ADME and pharmacokinetic aspects of these compounds. The *in silico* physicochemical characteristics, drug-likeness, and pharmacokinetics of sophia and camelina peptides are displayed in Table 5.10 and Figure 5.4, respectively. All these parameters were examined with reference to captopril, which serves as the standard medication. Similarities could be seen between the drug-likeness and pharmacokinetics of the peptides and those of inhibitor drugs such as captopril. According

to the data presented in Table 5.10, all the predicted dipeptides and tripeptides derived from sophia protein hydrolysates qualified as described by Lipinski. For instance, the rotatable bonds (RORB) of these peptides that were observed had a range of 0 to 9, and their TPSA values were lower than 130Å², which led to high gastrointestinal absorption. In addition, Table 10 shows all peptides (GF, SL, PM, M, PGL) as being highly soluble and had high values for GI absorption. In terms of camelina hydrolysates, Table 5.10 revealed all peptides without PEL, rotatable bonds observed ranged from 0 to 9. This is consistent with previous studies because compounds with more than 10 rotatable bonds show poor oral bioavailability (Ji et al., 2020). Furthermore, TPSA values of 2 dipeptides (GR, DM, QW) and 2 tripeptides (GGY, PEL) have higher than 130Å² leading to low gastrointestinal absorption. Table 5.10 shows all peptides were highly soluble and 17 out of 23 bioactive di- and tripeptide had high values for GI absorption, except for GR, QW, PQ, DM, GGY, PEL. This means that most of the peptides derived from camelina protein hydrolysates have very good absorption.

In addition, bioavailability six physicochemical properties, including lipophilicity (LIPO), molecular size (Size), polarity (Polar), insolubility (Insolu), flexibility (Flex), and instauration (Insatu), were observed when radar was displayed for a quick assessment of drug-likeness. Oral bioavailability is best achieved in the area colored in this Figure 5.4. According to Figure 5.4, all selected di- and tripeptides derived from sophia protein hydrolysates have their optimal values in the highlighted zone. Besides, the optimal values of most di- and tripeptides derived from camelina protein hydrolysates, except GR, DM, QW, GGY, and PEL, are in the colored region. As a result, peptides obtained from sophia and

camelina protein hydrolysates have the potential to exhibit drug-like properties which could be exploited in the pharmaceutical industry.

Table 5.10 *In silico* physicochemical properties and absorption, distribution, metabolism, excretion (ADME) profile of sophia and camelina bioactive peptides

Sample	Active Fragment Sequence	Physicochemical properties				Lipophilicity		Drug likeliness		Pharmacokinetics
		ROTB	HBA	HBD	TPSA (Å²)	ESOL	C LogP	Lipinski filter	Bioavailability score	GI absorption
Sophia hydrolysates	Captopril	4	3	1	96.41	-1.14 Very soluble	0.62	Yes (0)	0.56	High
	GF	6	4	3	92.42	0.32 Highly soluble	-0.24	Yes (0)	0.55	High
	SL	7	5	4	112.65	0.84 Highly soluble	-0.86	Yes (0)	0.55	High
	PM	7	4	3	103.73	1.04 Highly soluble	-0.32	Yes (0)	0.55	High
	GM	7	4	3	117.72	1.32	-0.82	Yes (0)	0.55	High

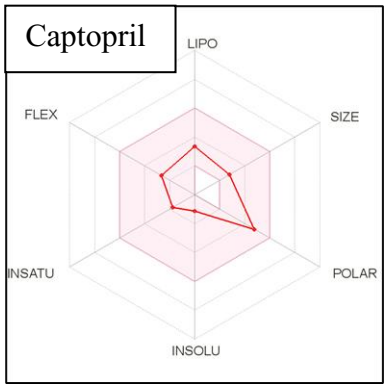
						Highly soluble				
	PGL	9	5	4	107.53	0.91 Highly soluble	-0.51	Yes (0)	0.55	High
Camelina hydrolysates	VF	7	4	3	92.42	0.11 Highly soluble	0.6	Yes (0)	0.55	High
	AL	6	4	3	92.42	1.05 Highly soluble	-0.41	Yes (0)	0.55	High
	VL	7	4	3	92.42	0.5 Highly soluble	0.27	Yes (0)	0.55	High
	GPL	8	5	3	112.73	0.99 Highly soluble	-0.6	Yes (0)	0.55	High
	AW	6	4	4	108.21	-0.09 Very soluble	0.11	Yes (0)	0.55	High

PF	6	4	3	78.43	0 High soluble	0.33	Yes (0)	0.55	High
PY	6	5	4	98.66	0.14 High soluble	0	Yes (0)	0.55	High
DF	8	6	4	129.72	0.83 Highly soluble	-0.59	Yes (0)	0.56	High
GL	6	4	3	92.42	0.86 Highly soluble	-0.49	Yes (0)	0.55	High
PM	7	4	3	103.73	1.04 Highly soluble	-0.32	Yes (0)	0.55	High
GR	8	5	5	156.82	2.31 Highly soluble	-2.16	Yes (0)	0.55	Low
PPL	8	5	3	98.74	0.09	0.06	Yes (0)	0.55	High

					Highly soluble				
IH	8	5	4	121.1	0.2 Highly soluble	-0.33	Yes (0)	0.55	High
QF	9	5	4	134.51	1.01 Highly soluble	-0.68	Yes (0)	0.55	High
QW	9	5	3	151.3	0.49 Highly soluble	-0.48	Yes (0)	0.55	Low
PQ	7	5	4	121.52	2.19 Highly soluble	-1.6	Yes (0)	0.55	Low
GGY	9	6	5	141.75	1.14 Highly soluble	-1.22	Yes (0)	0.55	Low
SF	7	5	4	112.65	1.03 Highly soluble	-0.77	Yes (0)	0.55	High

DM	9	6	4	155.02	1.67 Highly soluble	-1.2	Yes (0)	0.11	Low
IL	8	4	3	92.42	0.25 Highly soluble	0.63	Yes (0)	0.55	High
GW	6	4	4	108.21	0.23 Highly soluble	-0.21	Yes (0)	0.55	High
PL	6	4	3	78.43	0.66 Highly soluble	0.04	Yes (0)	0.55	High
PEL	12	7	5	144.83	0.92 Highly soluble	-0.52	Yes (0)	0.56	Low

Abbreviations: ROTB, number of rotatable bonds; HBA, number of hydrogen bond acceptors; HBD, number of hydrogen bond donors; TPSA, topological polar surface area; ESOL, estimate solubility; C LogP, logarithm of compound partition coefficient between n-octanol and water; GI absorption, gastrointestinal absorption.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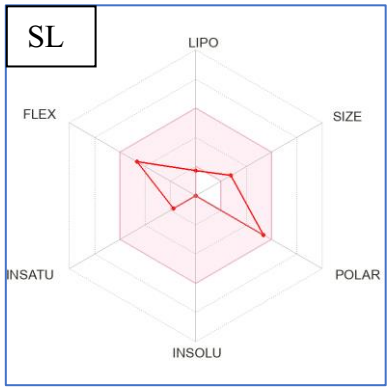
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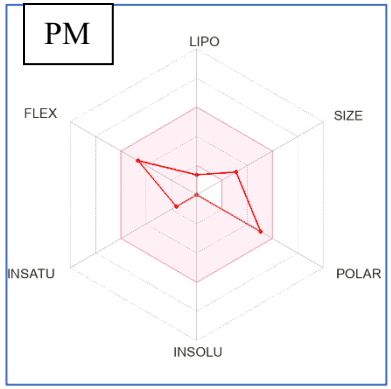
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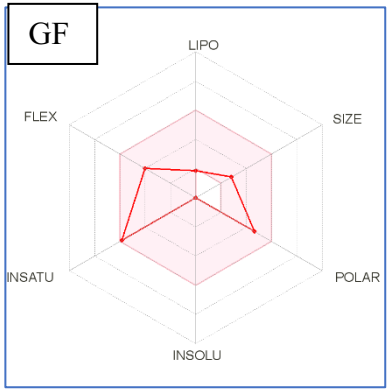
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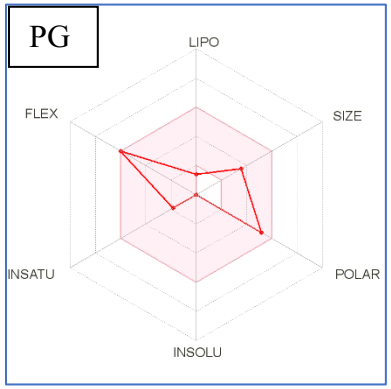
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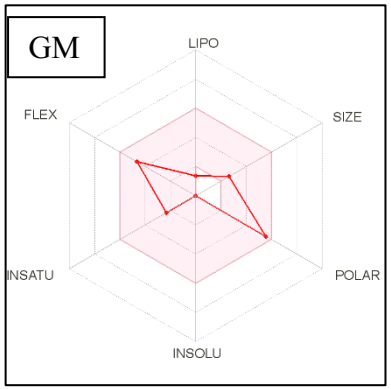
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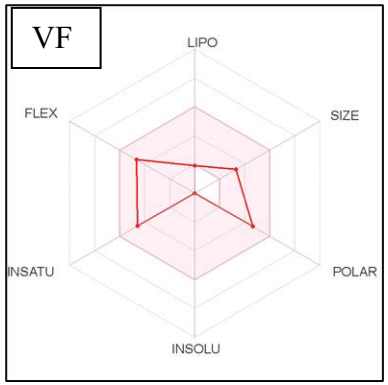
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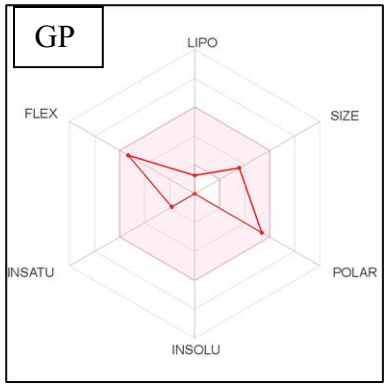
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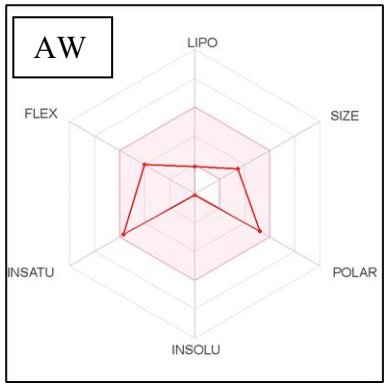
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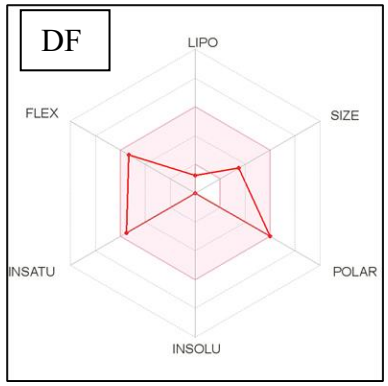
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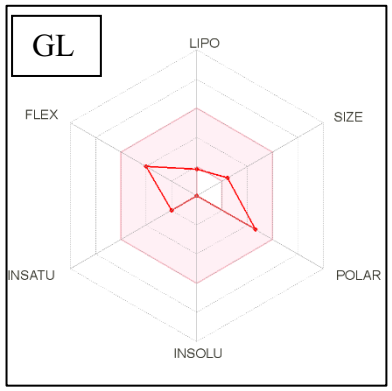
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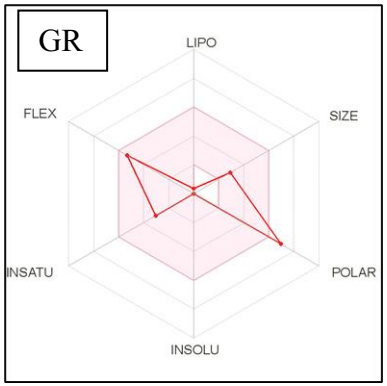
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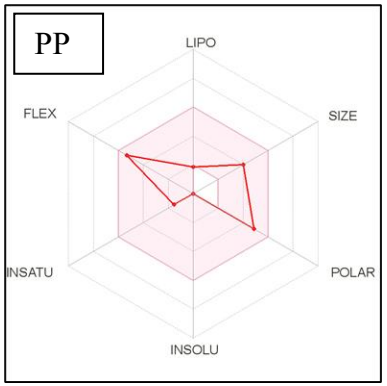
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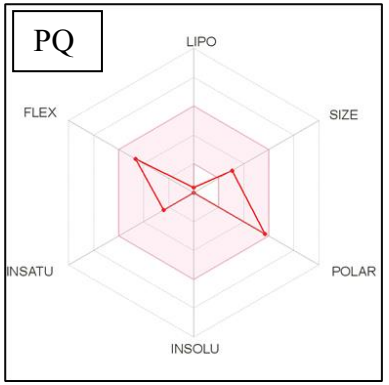
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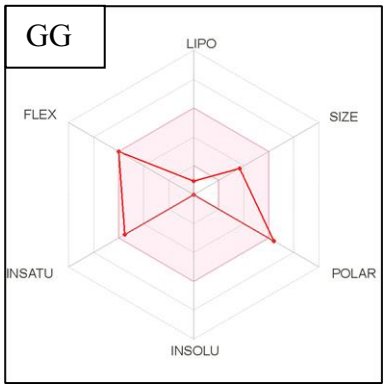
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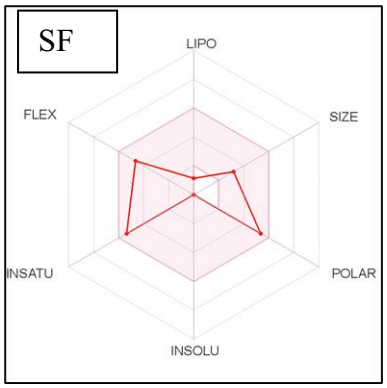
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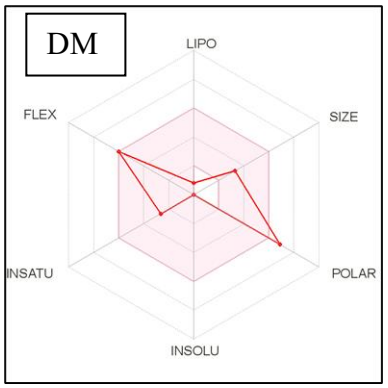
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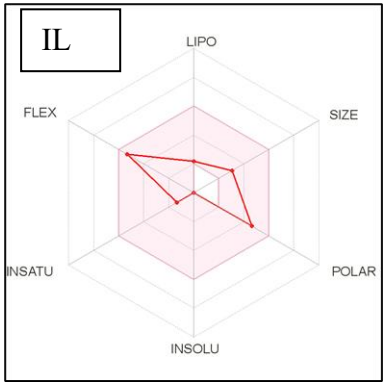
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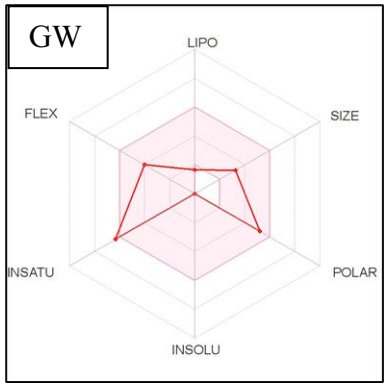
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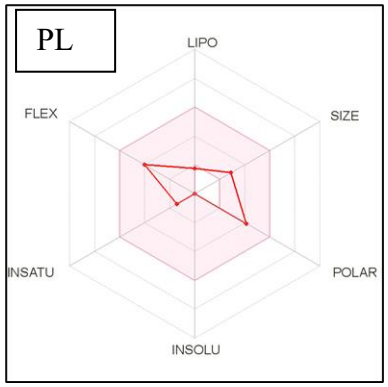
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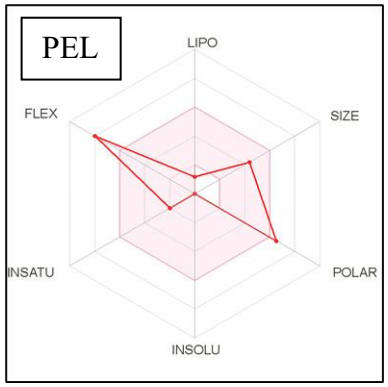
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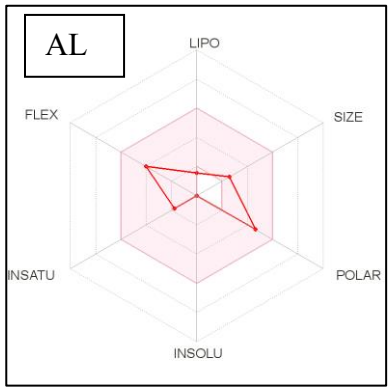
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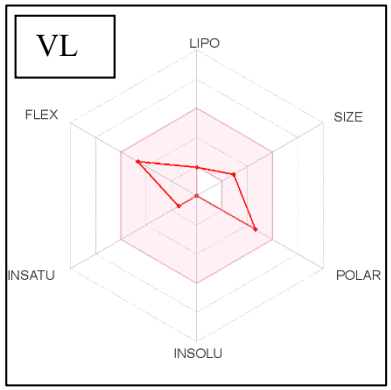
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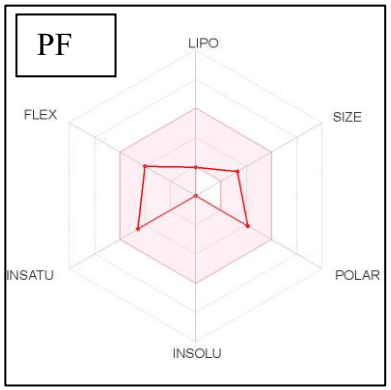
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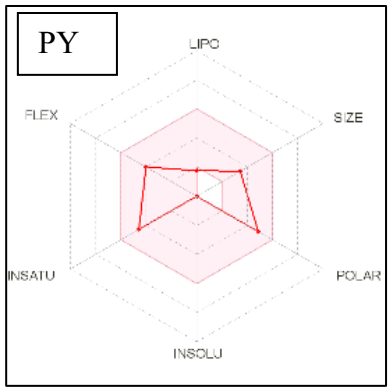
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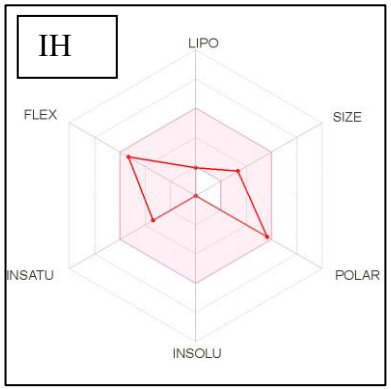
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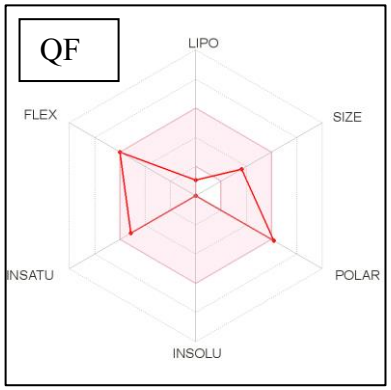
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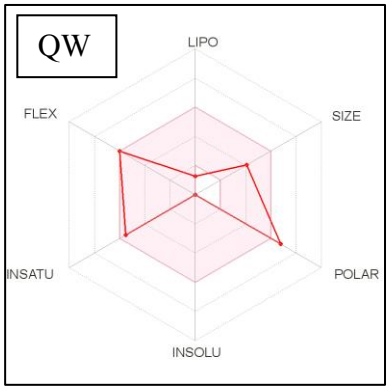
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Figure 5.4 Bioavailability radar of sophia-derived bioactive peptides and camelina-derived bioactive peptides and inhibitor drug (captopril) based on physicochemical indices ideal for oral bioavailability.

LIPO, Lipophilicity: -0.7 < XLOGP3 < +5.0; SIZE, Molecular size: 150 g/mol < mol. wt. < 500 g/mol; POLAR, Polarity: 20 Å2 < TPSA <130 Å2; INSOLU, Insolubility: 0 < Log S (ESOL) < 6; INSATU, Instauration: 0.25 < Fraction Csp3 < 1; FLEX, Flexibility: 0 < Number of rotatable bonds < 9. The colored zone is the suitable physicochemical space for oral bioavailability

5.5 Conclusion

This research demonstrated that sophia and camelina protein hydrolysates and their fractions was found to possess potent antioxidant effects *in vitro*. Moreover, the application of sophia and camelina protein hydrolysates resulted in the inhibition of human LDL cholesterol oxidation, as well as the scission of supercoiled plasmid DNA strands induced by peroxyl and hydroxyl radicals. In addition, peptides generated from camelina proteomes can be efficiently evaluated for their bioactive potential and physicochemical qualities using bioinformatics. BIOPEP analysis showed that camelina protein hydrolysates could be utilized as precursors for releasing peptides with dual functions in ACE and DPP IV inhibition. Furthermore, *in silico* toxicity prediction using Toxinpred revealed that all active fragment sequences released after GI digestion were nontoxic. The ADME analysis indicated that camelina-derived peptides have the potential for developing functional food and nutraceutical products. Therefore, the conditions for generating and processing bioactive

food protein hydrolysates need to be carefully designed to yield multifunctional peptides with diverse applications for maintaining optimum human health.

Author Contributions: Dr. Shahidi F proposed and supervised the project while Na T.T.N. conducted the experiments and analyzed data. Tha-rindu R.L Senadheera supported and helped analysis of the *in-silico* data. Authors Na T.T.N. drafted the manuscript while Dr. Shahidi F reviewed and edited the manuscript. The final document was read and approved by all author(s)

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Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 6

Summary and recommendations for future work

6.1 General overview

This thesis encompasses research that is primarily focused on examining the functional properties of protein isolates derived from camelina and sophia seed meals. Furthermore, it investigates the characteristics and bioactivity of hydrolysates obtained from camelina and sophia, as well as their corresponding fractions prepared using Alcalase and Flavourzyme enzymes. The overall aim of this study was to enhance the value of camelina and sophia seed meals by exploring their potential for producing valuable food and nutraceutical ingredients that are suitable for human consumption. Both camelina and sophia protein isolates, along with their hydrolysates, were thoroughly characterized in terms of physicochemical properties, functional attributes, and bioactive properties, both through *in vitro* and *in silico* analyses. This fundamental knowledge gained provides a basis for evaluating the utilization of by-product and these protein-rich meals in the food and nutraceutical industry.

6.2 Functionalities of camelina and sophia protein isolates

As mentioned in Chapter 3, this work compared the effectiveness of ultrasonic-assisted and traditional methods for extracting proteins from camelina and sophia seed meals. The findings revealed that the protein profiles derived from camelina and sophia protein isolates, extracted through the ultrasonic-assisted extraction technique, exhibited similarities to those obtained via conventional extraction methods. Additionally, the surface hydrophobicity of camelina protein isolates (CPI) and sophia protein isolates (SPI) was found to be higher than

that of soybean protein isolates. Notably, ultrasonic-assisted extraction significantly improved various functional properties of CPI and SPI, including solubility, water holding capacity, oil absorption capacity, emulsifying capacity, and foaming capacity. The findings from the study revealed that the water holding capacity (WHC) of camelina protein isolates (CPI) was higher compared to that of sophia protein isolates (SPI). Conversely, the oil absorption capacity and surface hydrophobicity of camelina protein isolates was found to be lower than that of sophia protein isolates. These findings also indicate the potential use of CPI and SPI as alternatives to soybean protein in food formulations. However, further investigations are required to evaluate the protein value and digestibility of CPI and SPI to determine their suitability as food ingredients.

6.3 Bioactive functionalities of camelina and sophia hydrolysates

As discussed in Chapters 4 and 5, the evaluation of camelina and sophia hydrolysates and their fractions prepared using Alcalase, Flavourzyme, and a combination of both enzymes indicated that those produced by Alcalase exhibited the highest potential antioxidant activities. Furthermore, it was observed that all camelina and sophia hydrolysates exhibited greater radical scavenging activity in both DPPH and ABTS assays compared to camelina and sophia protein isolates. Furthermore, it has been determined that the essential amino acid content of camelina and sophia hydrolysates is comparable and meets the recommended levels set by the World Health Organization (WHO), signifying their adequacy for human consumption. In addition, hydrolysates generated using Alcalase demonstrated the highest hydroxyl radical scavenging ability and oxygen radical absorbance capacity compared to other hydrolysates. The research findings indicated that camelina and sophia protein

hydrolysates exhibited an inhibitory effect on the oxidation of human LDL cholesterol. This effect was attributed to their ability to chelate copper ions and scavenge free radicals present in LDL. In addition, their hydrolysates inhibited peroxyl and hydroxyl radical-induced supercoiled plasmid DNA strand scission. In particular, hydrolysates produced by Alcalase and the combination of Alcalase and Flavourzyme demonstrated a more potent inhibitory effect on both hydroxyl radical-induced and peroxyl radical-induced oxidation compared to carnosine and date seed (Ambigaipalan and Shahidi, 2015).

According to Chapter 4, molecular weight was identified as a significant factor influencing the improved antioxidant activity in the study. It was observed that the antioxidant activities of fractions with a molecular weight of less than 3 kDa were generally higher compared to the hydrolysates and protein isolate. Particularly, the <3 kDa fraction obtained from Alcalase demonstrated the highest antioxidant properties. These findings suggest that the varying levels of activity observed in enzyme-derived hydrolysates and their purified fractions can be attributed to different peptide compositions, including their sequence and physicochemical properties. This phenomenon is consistent with other peptides derived from plant seeds such as hemp seed, canola seed, corn Gluten Meal (Alashi et al., 2014; Girgih, Udenigwe and Aluko 2011; Hu et al., 2020).

As mentioned in Chapter 5, bioinformatics can provide an efficient means to evaluate the bioactive potential and physicochemical qualities of peptides derived from camelina and sophia proteomes. By employing computational tools and algorithms, bioinformatics enables the analysis and prediction of peptide properties such as bioactivity, physicochemical characteristics, structural features, and potential interactions with biological targets. This

approach can save time and resources by prioritizing peptides with the most promising attributes for further experimental validation, thus facilitating the discovery and development of functional peptides from camelina and sophia hydrolysates. According to the analysis performed using BIOPEP, it was found that camel protein hydrolysates had a higher number of antioxidant peptides, ACE inhibitor peptides (angiotensin converting enzyme) and DDP IV inhibitor peptides (dipeptidyl peptidase IV) compared to sophia hydrolysates. The results suggest that camelina protein hydrolysates may have a greater capacity to exhibit antioxidant and inhibitory effects on ACE and DDP IV enzymes, which are associated with various physiological functions in the human body. Moreover, it was determined that all active fragment sequences released after gastrointestinal (GI) digestion of camelina/sophia-derived peptides were classified as non-toxic. Additionally, the ADME (absorption, distribution, metabolism, and excretion) analysis indicated that these peptides derived from camelina and sophia have the potential to be developed into functional food and nutraceutical products. These findings highlight the importance of carefully designing the conditions for generating and processing bioactive food protein hydrolysates to yield multifunctional peptides with diverse applications that can contribute to maintaining optimum human health.

6.4 Implications and recommendations for future work

As the global population continues to grow, there is an increasing reliance on plant proteins as a sustainable and alternative source to animal-derived proteins. The excessive production of reactive oxygen species and oxidative stress is known to contribute to the development of various diseases. Therefore, understanding and addressing oxidative stress are crucial for preventing and managing these conditions. To effectively utilize plant proteins, it is essential

for the food and processing industries to comprehend their physicochemical properties, structural characteristics, amino acid composition, and functional attributes. This knowledge enables the development of innovative plant-based products that provide diverse nutritional benefits, promote healthier dietary choices, reduce dependence on animal-based proteins, and address sustainability concerns in the food industry.

Bioactive peptides derived from camelina and sophia protein hydrolysates hold great potential as both food and preventative agents for managing various health conditions. These multifunctional bioactive peptides have demonstrated the ability to attenuate or control blood pressure and oxidative stress. Additionally, it is important to investigate whether these peptides possess bioactive properties that could contribute to their observed antihypertensive and anti-diabetic effects *in vivo*. By exploring the bioactivity of these peptides, further insights can be gained regarding their therapeutic potential in managing and preventing various health conditions. By utilizing these findings, the incorporation of camelina protein isolate and its hydrolysates in high-value applications such as functional foods and nutraceuticals can significantly increase the value-added utilization of camelina and sophia meal and boost the profitability of the food industry.

There are exciting opportunities yet to be explored in the production of bioactive peptides. The research findings indicate that enzymatically hydrolyzed camelina and sophia proteins possess bioactive properties that have the potential for development in the food and nutraceutical industry. The use of alternative protein sources such as those derived from camelina and sophia seed meals have garnered increased interest due to their potential benefits in food formulation. These proteins can offer unique functionalities including

solubility, emulsifying, foaming, water absorption, and other properties, which are all essential characteristics in a range of food products. However, to fully exploit the potential of these protein isolates incorporated in food formulation, more research is indeed necessary. Further purification is needed to identify the specific amino acid sequences responsible for the various bioactive properties, allowing for a better understanding of the structure-function relationships of the peptides and enhancing their use in the nutraceutical industry. To validate their efficacy and safety, clinical trials will be necessary to support the data obtained from *in vitro* and animal studies before the products can be utilized as preventive or treatment options in humans. These explorations and trials offer promising prospects for developing innovative bioactive peptide products with potential health benefits. It's important to note that regulatory aspects also need to be considered. Before they can be included in food formulations, they must comply with food safety standards and regulations established by food safety authorities such as the FDA in the United States or the EFSA in the European Union. This includes proving that they are safe for consumption and accurately labeling them on food packaging. The exploration of these novel protein sources represents an exciting area of food science, promising both environmental sustainability and potential health benefits. However, it's a field that requires careful study and rigorous testing to ensure the best outcomes for both the industry and the consume.

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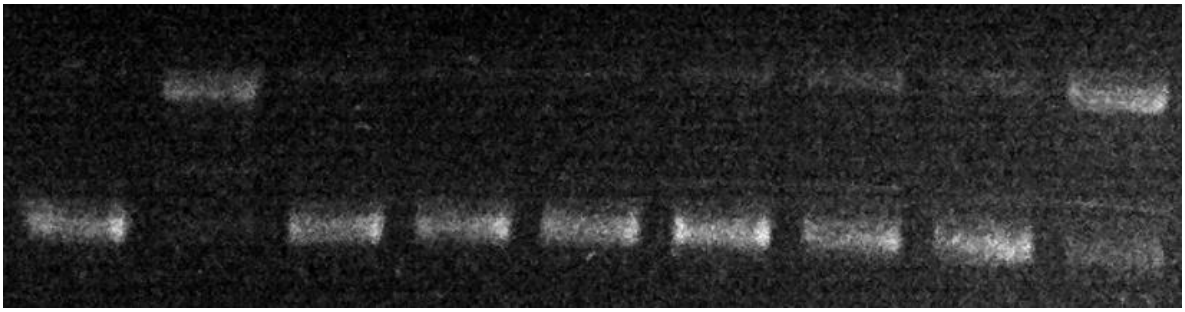
APPENDIX

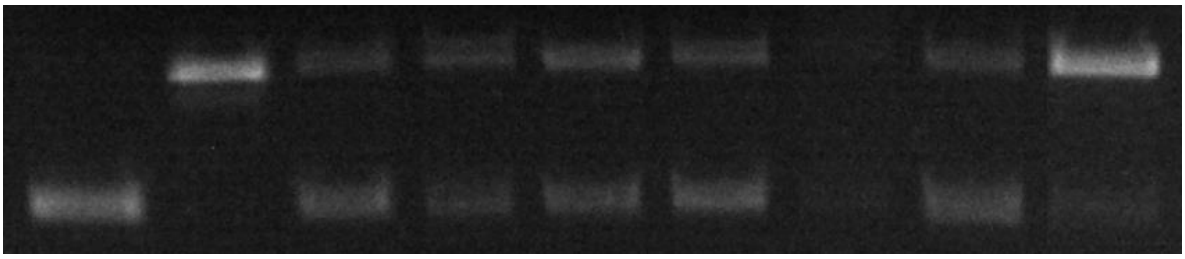
Appendix 5.1 Original results of peroxyl and hydroxyl radical-induced supercoiled DNA strand scission

- Inhibition of peroxyl radical-induced supercoiled DNA strand scission



Blank Control Ca_AL Ca_FL Ca_ALFL So_AL So_FL So_ALFL Carnosine

- Inhibition of hydroxyl radical-induced supercoiled DNA strand scission



Blank Control Ca_AL Ca_FL Ca_ALFL So_AL So_FL So_ALFL Carnosine

Appendix 5.2. Identified peptides from camelina protein hydrolysates using LC-MS/MS

IVNDN(+.98)GDRVFDGQ
IVNDNGDRVFDGQ
AWPDKNPFFPSDPY
NLDDPSDADVYKPSL
NHPGQIGN(+.98)GYAPVL
VITN(+.98)GFQISPQ
ETTLTHSSGPASY
VDDVHYYDPVQDK
VEHIRSGDTIATTPGVAQ
IVNDNGDRVFDGQVSQGQ
YDDIANNPLNPRPGTL
VNDNGDRVFDQ
VNDN(+.98)GDRVFDQ
INHPN(+.98)GDDVYAGVPK
TFIESPVFGE
TLPSITPPGTY
SGDVWDPQPAPK
HQKVEHIRSGDTIATTPGVAQ
VTPGSDSPKVSPE
NLDDPSDADVY
TKGKYPDIM
HAGKAPTNDRFDDL
NHPGQIGNGY
GPFGVIRPPL
KIDLQTAQ
FNAPIYL
PPPPGAL
EMPVPPAQ
NLGGTVSVK
V(+42.01)SVSPSVL
A(+42.01)DVAAVSAGN(+.98)DYE
G(+42.01)YGSPTTTTQGPQ(+.98)
NPAAGN(+.98)PSLQ
IVGKN(+.98)VPL
FVDGKTK
TCGVGSLSL
SRAPATGL
PSSQGGGPSPGLSVFQ(+.98)
GGVAPVGSAIAL
DPGQ(+.98)GGGFGMKAM
SIGTTVVNE
A(+42.01)SSAAAGSL
N(+42.01)GGGGGHPQDGK
SAAAGGNHVPE
N(+42.01)GGGGGGGGGGGGGGGGGGGPPK
TM(+15.99)LKRRVPPAVPGIM(+15.99)
A(+42.01)N(+.98)N(+.98)SGLCGVPLL
SSSSSSLSK

NHPGQIGN(+.98)GY
DGEWTAPTIPNPE
DWYKGPTLL
IVNDNGDRVFDGQVSQ
EDPLLGDVQ
N(+.98)GGTIEIPHPL
YVTDGEAQVQ
DMVGPIVEQPE
NNPVLIGEPGVGK
NNPVIIGEPGVGK
NGLVPIVEPE
SPVFGESQGQGQ
DDPSDADVYKPSL
IVGDDLLVTNPK
LVGDDLLVTNPK
DDPSRADVY
NALEPSHVL
SHSQTLPSITPPGTY
ITIPVNIPGQFQ
GPDHGIHPL
RIIPGYGGGM
EVITN(+.98)GFQ
IPSGPSY
MNPIGKVPVL
DLVGPIVEQPE
AGRTSVLRGLPL
KNIFSGFGPEVIAQ
NSAAGPIFSVK
FGGNGGKL
T(+42.01)VTNQ(+.98)N(+.98)SVGPSVGRVRGGQ
LRVGPPL
KN(+.98)ANLGGGPAKN(+.98)GGK
A(+42.01)N(+.98)GAVTTTPASNGQ(+.98)
NGGGGGGGGGGGPPKMVL
FN(+.98)AFATN(+.98)VAM(+15.99)
FLTGGGGAL
SN(+.98)AVGN(+.98)LGLN(+.98)SAYGDAL
THLVSCGGK
PFALVALVL
S(+42.01)GTSM(+15.99)AAPAISGVVAL
G(+42.01)TAAPLGIPQ
YTGGASGN(+.98)LAASE
G(+42.01)GGGGLGGGGGGGL
PVIITACE
ITGAPQIGN(+.98)TY
YVPPKAPHDL
P(+42.01)LGSDDGVQ(+.98)PPE
KDGTTTAGNSSQ(+.98)
GSSPGSILPGIGNTGM(+15.99)
T(+42.01)SSYVASTSKGILSDSM
SRGASFAMAGAVVLGSQ
LDSGTSM(+15.99)ATPVVSGIIALL
AILYKSVGIASVATL
GADQPVIL
GDSHGGGGGDE
GGRFGVDK

VVRPPLRQPYE
NPIGKVPVL
IGKGKIPDPGSL
NSGGGGGGWKGGGGQGGGWKGGGGQ
DVAPPRGPL
VWDHHAPQL
NQLPIVGPL
NQLPLVGPL
DDPSRADVYKPQ
KMNPIGKVPVL
VTDGEAQVQ
FVSVGTAVGFAGL
HDLPGHL
AVITN(+.98)GFQISHEE
THHPSFGGN(+.98)GATPL
IDVPNGRL
NALEPSQVIQ
KNIFSGFGPE
IGTPGKGIL
DAGPVERPIL
DRNLRPVL
FTPPVNVE
DDVGYGGY
RGSLGIIAPL
RGSIGIIAPL
VVRPPLRQPY
KSSGGGGGGGGGGGM(+15.99)
TEAPLNPK
VIGGGVHPL
PEVGSSIN(+.98)K
VDGSGSPGVK
ASSSSSALK
VN(+.98)DNGDRVFDQ
NIPDNKPAGE
AHLGPGKL
TSSISAVK
N(+.98)GVPPGGGFE
M(+15.99)SVVASAPGKVLM
EPHNVSVDE
ARFAATL
A(+42.01)KEPVRVL
IAIGTPPQ
DEDVYGGY
FDNAATTVM
SGGGGGANTAGL
N(+.98)GGGGGGGGGGGGGGGGGGGGPPKM(+15.99)
IQ(+.98)SHGEN(+.98)Q(+.98)
T(+42.01)GASAMSPK
LSSMGSSGIL
N(+.98)SSSINPNDLN(+.98)ILNL
H(+42.01)SKGGGGGGGGN(+.98)K
VGSIGAGGRY
DAGQTIEDK
GGSPGIGGGL
FVSVGTAVGFAGLAAY
M(+15.99)N(+.98)GHHGGGGGGPK

YEGPSTKNPL
IDVPN(+.98)GRL
ISDPSGIFPQ
N(+.98)GLVPIVEPE
N(+.98)LDDPSDADVY
LMSGGSGGGVDE
IIHVVPQ
VN(+.98)GPANGSAPL
MAPHMNPTATEY
HIRSGDTIATTPGVAQ
V(+42.01)SGDATSGGGGGGGGGK

GDDPDAVIPGL
AVITN(+.98)GFE
AGFDPDTL
HGNPVQL
NNGNEPLIL
NIFSGFGPE
KTGPGLGSGPSAGPN(+.98)L
AVFAGVPVGGTL
N(+.98)AGPNGGKKGGGGGGGGPM(+15.99)
YNNGNEPLIL
AIGDVIPGPM
G(+42.01)CSAGVIAIDLAK
KIGEGPVAQ
VTPRVPE
P(+42.01)NPTPVSVVPK
VITN(+.98)GFQISPQE
V(+42.01)AGFCAQ(+.98)AL
Y(+42.01)GPDPIIS
EVSSTSVDDQ
QPQQVTGGTAL
IVKGSSGQ(+.98)Q(+.98)
RVGN(+.98)GLEE
DVATISAIPL
NGGGGGHPQ
VLPGSIGDMDTLRNL
L(+42.01)TEIICGSRSDPY
TAAAAGASTSAQ
AN(+.98)GN(+.98)GNN(+.98)N(+.98)IN(+.98)GFSVASGG
Q
KYRSAGTL
VVRPPLRQ(+.98)PYE
IDPKRGSLGIIAPL
S(+42.01)PGGGMNSSSGHISPGAGFL
G(+42.01)DGLGPVVQ
N(+42.01)VSPCSVAVLVDQ(+.98)
Y(+42.01)TGGASGDSAASE
PNGGSEDGAPSQPSK
R(+42.01)GGIVGAAALTGGTLM
DKTASTPL
TCSSTNCDE
N(+.98)(+42.01)VTSSVGIIM
DVWDPFE
GLITGSDTE
GASKEDIEQ

AASAAAATTK
AEAGGSPAL
MVNDNGDRVFDQ
RVKFNTL
DDPSDADVY
VNDNGDRVFDQE
SVTVPKVPE
WFYNDGQQPL
IYIPLPDEDSRL
GHDPSRQIE
LGVLASYGGQ(+.98)
NKIRVPYQPIDAQ
VITN(+.98)GFQ
NYPPVYPVGPVL
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VNDN(+.98)GDRVFDQE
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SYLPDLTE
G(+42.01)GGGQGGHK
L(+42.01)GVAGAGVLAGYDQ(+.98)
I(+42.01)VNPPPNVGVGDDDTE
G(+42.01)GHKGGGGGGGQ(+.98)
W(+42.01)ANALGATVIGTVSTNE
LFGGGGSN
VTSESSDLGTGE
FKVPGNENPSIL
WTAFGAL
SGGMVLPQ
LNTIN(+.98)SAGGFL
WAPCAAHCIDQM(+15.99)
RSPPPVQ
N(+.98)(+42.01)GGGGGGGGGGGGGGGGGGGGPPK
SSSLSRL
FPSGVGTL
ITPFPAGPL
G(+42.01)GTCAIPGAFGCGK
GASAVGLTAAVHK
PQVN(+.98)ASAPGSIGFTPM
VNQPNFK
GNAAPAGPDAGPRTMK
PMPSPPAPVSGGVN(+.98)VIQ
N(+.98)(+42.01)AGASADIKL
ISTPIINPPQ
VFGGGISTL
VSQGQLIVVPQ
SVRTPTSSSFVVGAISE
PN(+.98)VAETIGAFAFTL
LTEAHSTINSLE
G(+42.01)SGSSALSK
PSVASYFL
A(+42.01)GATALGVTM(+15.99)
Y(+42.01)GSLFITTFE
K(+42.01)GITVVCSGGN(+.98)SGPAAQ(+.98)
ENGFPVKGGGK
N(+.98)(+42.01)CVVRGNL
GHRGISGGE
LHASNGFSGAEISPE
ALTAGVAAAAL
EVLIQGGVIDNL
VYLLATE
PHNVSVDE
IDVRGPEL

HSSVGTGSL
S(+42.01)VDIIGFGTGPAL
P(+42.01)Q(+.98)MGGGPGPGPM(+15.99)
T(+42.01)NPAVGMN(+.98)GMSGNE
L(+42.01)PGGGAGGVGGGK
IEFPLPDIK
MKPAPVK
D(+42.01)LPPGVL
KPTGEGGGGGGE
FLGDVPK
DIPPPRGPL
NPVSGSGHVE
VNN(+.98)VGM(+15.99)IGSE
I(+42.01)SMSGGSQL
CAPGNAGISSSGDATCIPDL
LDRGHSN(+.98)SLSGATE
VNGSQVVAGDEHGGGNK
DIGVSHGMQ
SRASVKNNVITEGN(+.98)L
RIQPVNY
KEGAGPVNL
RDNPNEPL
Q(+42.01)GGEAGGPAPGGMDE
N(+42.01)GGGGGGGGGGGGGGGGGGGGPPKM
CIGSGGACGYN(+.98)K
G(+42.01)Q(+.98)KAQ(+.98)AATAAGGSSTE
V(+42.01)LVAGAN(+.98)CFL
SLSINN(+.98)LVGE
G(+42.01)N(+.98)VSFRVTDN(+.98)E
V(+42.01)HETGACISGGSSISLL
DFSIFSPL
EEGGDSN(+.98)AAAAK
ARGGVSTNNL
TEGSGVAIEGPSE
V(+42.01)AAGVSFCAAL
GGPKGEIAY
S(+42.01)VPSIPQSLNQQ(+.98)
ADELSSLSPGQ(+.98)
N(+42.01)AIRNDHSK
ITNVPSRYVRSDE
L(+42.01)SGAGTPPL
S(+42.01)PNPASMVLDAIEGSK
S(+42.01)VETVNNL
GGGGGGSRARE
APPGGAGNDNKN(+.98)Q(+.98)
FDVGSN(+.98)SL
K(+42.01)PVPVPTTP
N(+.98)ISFTVWDVGGQ(+.98)
I(+42.01)PTFVADPL
IDVPNSRL
V(+42.01)SAVGDDAFSN(+.98)VK
RSGGGGGGGE
LPNVSDPGSAGPIYQPSM
A(+42.01)SAN(+.98)VLAK
ISFAAMVQ(+.98)
NGGGGPGPAGGK

E(+42.01)DEGGTAGE
AFGGGGFAL
EISN(+.98)GQL
S(+42.01)VCGPAGRLL
N(+.98)(+42.01)GLAAAGAAGPL
IHPIPPL
ELVVVCDACE
SIAAVGGVE
FGWDKDLAK
FDTGSSN(+.98)L
SPVASSSGGSTVIE
K(+42.01)PSGAGVNPSSSSE
IQRGISFPN(+.98)C
V(+42.01)ALGCVVSSGSM(+15.99)
HN(+.98)LSISSFLL
VHAAPGGGSSLGY
NSSGTSN(+.98)DGVLL
IVVFDPL
EN(+.98)GLVPIVEPE
RTVGADVL
Q(+.98)VGTIAAL
A(+42.01)SNISNVM(+15.99)N(+.98)GQ
K(+42.01)VAAHLN(+.98)E
IDEPSFTQPK
SSSIQASVK
M(+15.99)DGGGGPNQTIL
RDGVKSCCML
FQ(+.98)SDVLLIPL
G(+42.01)GGGQAQPPQ(+.98)
AGAATGGFL
PSGAGGE
EVITNGFQ(+.98)ISPQ
Y(+42.01)SASGGGGGGGE
YALSSALGL
N(+.98)SLVPLVACFVTAPM(+15.99)
APKNGGGGGGGGGGGGGGGGGGGPPKM
AGTAVISLM
DAGAISGLNVL
ASIGPGVGQGTAAGQ(+.98)
KAAAAAHGGGNGGGQ(+.98)
ADAASAPAYL
GWIAGPAAL
QQPWANQ
VGSSPFAPSSL
C(+42.01)PITGFL
MFGGGRGPM(+15.99)GGGGGML
Q(+.98)QKAVPHSSQ(+.98)
EQDVHGGGGSSK
SDGDSASSIL
SLGGGGGACYQ(+.98)
VRSSSGTTTSAAQ
S(+42.01)AAPAGNPMGN(+.98)SGYGF
VDAFACRPE
GDGVGGGGGGGGGSGSSN(+.98)GSGFGSLGRK
EVITN(+.98)GFQISPQ
PAPLAGCE

NIGGVSYGN(+.98)AGGGSHNGGGCVIGL
SN(+.98)GGIGGQ
S(+42.01)DSSNSSGAVL
DAASMAAGVSSPK
I(+42.01)VDQGSTSCGTAGAL
LN(+.98)DFN(+.98)GTNAN(+.98)VLM
VNNSNPSIGGAGGFGGGM(+15.99)GQSM(+15.99)
VVVPGGGVHPL
VDIGGGLGTIM(+15.99)
GPPSGGGGGGGGGGGGK
IHN(+.98)IFGGADDQ
FGGYAPGILSPSPAML
VNVSVVGDE
G(+42.01)NN(+.98)SLTGN(+.98)VPHEL
SDVDPVQ
WTAPTIPNPE
FGGGNLPAFVL
FGGGNIPAFVL

Appendix 5.3. Identified peptides from sophia protein hydrolysates using LC-MS/MS

HIEPVPGEE
LGVPIVM
T(+42.01)GGFTAN(+.98)TSL
RADGPAGIL
FLTIGPGDFL
PIDAVFM
N(+.98)(+42.01)ATAGGIISKIL
RAPWLEPL
NKTPHVSVCGDVK
INGGTISPFL
M(+42.01)GVAGVLGAALL
EVLPRGNAL
LN(+.98)GIPVAL
ASIGPGVGQGTAAGQ
A(+42.01)IGTAN(+.98)PANHVLQ(+.98)
A(+42.01)IGTAN(+.98)PANHVIQ(+.98)
PGVLPVASGGIHVWHM(+15.99)PAL
T(+42.01)SNPNVGGFGQ(+.98)GDAEIVLQ
PIIGIGIY
Q(+.98)(+42.01)ATATSISL
M(+15.99)TVTGCDDPRSL
ISTGAGAIRE
ICLCFGAKISE
A(+42.01)VGDTVAFAL
DN(+.98)GDGIAVGWL
W(+42.01)AQASQ(+.98)VIQ
DFTTQ(+.98)AAL
VRN(+.98)SMASVN(+.98)QML

MIAAGN(+.98)SLALNRGIQ(+.98)
S(+42.01)ATEAQ(+.98)AHGIVDL
ETKDTDIL
LPMITGFM
TDAVQTGTGQ
TKTFQGPPHGIQ
LSCSAGSVAQ(+.98)
PVPGEETQ(+.98)FIAY
ERDTISPSSIE
SNSHFVAAAQL
LTGIPVASTL
NLAPSPPHSL
WYTICICIL
M(+42.01)GGASSSIL
LTGGFTAN(+.98)TSLSHY
N(+42.01)LRAAHK
DILEAHKGPFTGQ(+.98)
M(+42.01)AGASSLDEIRK
G(+42.01)LITSRN(+.98)M
TM(+15.99)LNTLDGVRDE
I(+42.01)SMPAHLM
ICGTIAADE
WDPSFIQ(+.98)
SLCGIPPL
GRPLLGCTIKPK
FVSRFAPDQ(+.98)
NVSTPLTTPIVE
S(+42.01)VFTSIPDL
SQPITFL
LGDGIVSSDAE
S(+42.01)N(+.98)IVGIQ(+.98)ESVHE
G(+42.01)TAAGQAVEGIARQ(+.98)
W(+42.01)YTICICILEL
EVHVQVLHSM(+15.99)
NPFVAKHLL
IHGRSSGHY
LGGFHPQ
N(+.98)PDSSSHL
NKTPHVSVCGDVKL
CSEIVSAGTDTSATTL
G(+42.01)RSPTHGDLVE
LTGGFTANTSL
GDGKEGTSTIPGFNQ
DQ(+.98)PLTSNPN(+.98)VGGFGQ(+.98)
PESSAIM
ADGAATVGGE
ILADGAATVGGE
N(+42.01)SVISTTL
FIDPLTSIM
LSLAPSHQ
D(+42.01)SGVPAIAEHEGK
WAISLQNCAQFHM
TWVQDPL
YAIQVLDE
T(+42.01)WGGGELVAVGGK
V(+42.01)RSIDSISL

VTSSNLIQ
TESIRRL
DDN(+.98)AAVVIDQEGNPK
ISKN(+.98)CSATVGQVGNVGVNQ
N(+.98)(+42.01)EIFPNM
SFSGARGNASQ
YTAGSSIFL
P(+42.01)LGTAIHN(+.98)IE
LVCLSFVM(+15.99)GPIQ(+.98)
RIFIGNIPLM
PMITGFM
LYCTAIGAL
G(+42.01)HPWGNAPGAVAN(+.98)RVAL
IAAGNSLALN(+.98)RGIQ
WLSSGGLGAM
GTAAGQAVEGIARQ
IHNDTM(+15.99)SALGRPQ
P(+42.01)DAMEGPTPISAL
IFTMFTIL
I(+42.01)VTGVPDAIPVIGSPL
W(+42.01)IQ(+.98)N(+.98)THALAPGVTAPGE
S(+42.01)TCMLLAGIL
N(+.98)N(+.98)RVNPL
RTN(+.98)FGIGHSIKDL
SRALSCSAVQ
R(+42.01)AVFARE
T(+42.01)SPTTGGVTASFGML
PDSDGAIDGHL
DFSVETLEASN(+.98)L
AETGEIK
DQAISVY
RIRSVADLLQ(+.98)DQ(+.98)
LKDVPGL
LGMLPGL
I(+42.01)GEPADPFATPLE
CAYDVAPGGLL
M(+15.99)PIGVPK
A(+42.01)FTRGGAL
IDEPDLAN(+.98)LPY
N(+42.01)HGMHFRVLAK
GSLGDSVGN(+.98)FL
TFGEAIPPQ
C(+42.01)TAIGAL
AVGLASIGPGVGQ(+.98)
VCSSGGARM
SIGTALDNCL
SLPWDQIGY
NWGLN(+.98)VSTPL
RTN(+.98)VN(+.98)GDTGAGDRKKE
PSHPSSSKSPL
GLGSLSWAGHQ
S(+42.01)FGSSNIN(+.98)RL
V(+42.01)ALIGAISVARQ
G(+42.01)TTIIGGTIPK
MFLFSGRGY
LN(+.98)HHLAGLL

QRIFIGNIPL
Q(+42.01)MAKISSVL
LNGIPVAL
FQHWIGVGLE
M(+15.99)VSVPAGLL
TTSWHAQLSL
AINWGLSGPML
QIISHGFIGAAL
L(+42.01)IMNNTKRASFFCK
W(+42.01)DWLRGAMATVDRDY
M(+15.99)(+42.01)GVAGVLGAALL
GPTPISAL
NVGAVLIL
P(+42.01)SGHKVE
NWSNGSK
A(+42.01)LFSDGAAAL
CETFLNDIQ
ICGTIAADEK
P(+42.01)GVPPEEAGAAVAAE
V(+42.01)SNIFISNDFPQ
GYVATSRDPIIGGSLLL
GEPAWPN(+.98)DLLY
LDTPGPY
A(+42.01)VGLASIGPGVGQ
HLASNIGVAKSQ
AGASSLDE
FIAYVAYPL
GTIGIAL
SKAMDSSAPIE
N(+.98)IPQN(+.98)N(+.98)IFL
AM(+15.99)DSSAPIE
FIDPLTSIMSIL
GCTIKPKL
Y(+42.01)IFQ(+.98)VINSY
IVSAGTDTSATTL
N(+.98)(+42.01)LESNN(+.98)VGVVL
I(+42.01)PGIGHNL
DSTSTML
ARN(+.98)EGRDLAVE
AASTSPGQ(+.98)
AVGWPLIIY
I(+42.01)FQ(+.98)VINSYLIDE
CLGGITL
I(+42.01)TIFVAPECFSLCSYL
HLRTINAN(+.98)IGM
GEGVLDPQ(+.98)
IPSTSLL
LFFLGSVVL
A(+42.01)IGTANPANHVLQ
A(+42.01)IGTANPANHVIQ
ATGRIVCAN(+.98)CHL
VTSSNLIQ(+.98)
FDGTPWY
VCLNFTMGPIQM
S(+42.01)SGGLGAMGFGL
LFAN(+.98)PFV

K(+42.01)SPVSSQ	VAAGIFL
A(+42.01)VVKIPY	T(+42.01)STACWTAMAALL
I(+42.01)GAFGSGKSRAQ(+.98)	QFGGGTL
MVAGRDTTSSTL	A(+42.01)SIGPGVGQ
FCAEAIY	S(+42.01)DTSSILM
NGDVGGGFRGIQ	N(+42.01)SDIDPQ(+.98)
A(+42.01)SIGPGVGQ(+.98)GTAAGQ(+.98)AVE	ACVQARNEGRDL
M(+42.01)GGM(+15.99)AISIPK	NNRGARVL
VLLSAMGSNL	GHPWGN(+.98)APGAVANRVALE
TIGPGDFL	TGIPVASTLM
R(+42.01)TN(+.98)VN(+.98)GDTGAGDRKK	PDGSKSNNTVY
HLRTIN(+.98)AN(+.98)IGM	A(+42.01)VGLASIGPGVGQGTAAGQ
IVFSGNGDTL	MNLNEIVL

(Letters inside the parentheses indicate posttranslational modifications including (+42.01): acetylation; (+15.99): oxidation;

(+0.98): deamidation)