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Thème

Les graines de pin pignon, une source alternative de protéines pour l'industrie pharmaceutique et agroalimentaire

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Abbreviations list

$\mathcal{A}$

AAPH: 2,2'-Azobis(2-amidinopropane) dihydrochloride
ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid
ACE: Angiotensin-I converting enzyme
AE: Alkaline extraction
AE/IP: Alkaline extraction and isoelectric precipitation
AOAC: Association of Official Analytical Chemists
AUC: Area under the decay

$\mathcal{B}$

BBD: Box-Behnken design
BHA: Butylated hydroxyanisole
BHT: Butylated hydroxytoluene
BSA: bovine serum albumin
BV: Biological value

$\mathcal{C}$

$CaCl_2$: Calcium chloride
CCK-A: cholecystokinin A
CH_2: methylene
CH_3: methyl
CV: Coefficient value

$\mathcal{D}$

DF: Defatted flour
DH: Degree of hydrolysis
DNA: Deoxyribonucleic acid
DPPH: 2,2-diphenyl-1-picrylhydrazyl
DPNF: Defatted pine nut flour

$\mathcal{E}$

EAI: Emulsifying activity index
EC: Emulsion capacity
ELISA: Enzyme-linked Immunosorbent Assay
E/S: enzyme-to-substrate
ES: Emulsion stability
ESI: Emulsion stability index

$\mathcal{F}$

FAO: Food and Agriculture Organization
FC: Foaming capacity
Fe^{2+}: Ferrous iron
Fe^{3+}: Ferric ion
$FeCl_2$: Ferreous chloride
$FeSO_4$: Iron (II) sulfate
FRAP: Ferric reducing antioxidant power
FS: Foaming stability

FTIR: Fourier-transform infrared

$\mathcal{H}$

H_2O_2: Hydrogen peroxide
HCl: Hydrochloric acid
HPLC-MS/MS: High-performance liquid chromatography tandem mass spectrometry

$\mathcal{I}$

IC_{50}: Half-maximal inhibitory concentration
IgE: Immunoglobulin E
INFOGEST: International network of excellence on the fate of food in the gastrointestinal tract
iNOS: Nitric oxide synthase
IUIS: International Union of Immunological societies

$\mathcal{K}$

KCl: Potassium chloride

$\mathcal{L}$

LAB: Lactic acid bacteria
LPS: Lipopolysaccharide

$\mathcal{M}$

MAPK: Mitogen-activated protein kinase
2-ME: 2-Mercaptoethanol
Mw: Molecular weight

$\mathcal{N}$

N: Nitrogen
NaCl: Sodium chloride
NAD: Nicotinamide adenine dinucleotide
$NaHCO_3$: Sodium bicarbonate
NaOH: Sodium hydroxide
NF-kB: Nuclear factor-kB
NK: Natural killer
NO: Nitric oxide
NPU: Net protein utilization

$\mathcal{O}$

OAC: Oil absorption capacity
OD: Optical density
-OH: Hydroxyl group
·OH: Hydroxyl radicals
OHC: Oil holding capacity
OPPPI: Optimized *Pinus pinea* L. protein isolate
ORAC: Oxygen radical absorbance capacity

$\mathcal{P}$

P/A H: Pepsin/alcalde hydrolysate
PBST: Phosphate buffered saline with Tween-20
PC: Protein content
PDCAAS: Protein digestibility-corrected amino acid score
pepT1: Peptide transporter 1

PepT2: Peptide transporter 2
PH: Pepsin hydrolysate
PI: Protein isolate
PID: Protein isolate digestates obtained during intestinal digestion
PIG: Protein isolate digestates obtained during gastric digestion
PIL: Wholemeal pine nut flour
PILG: Wholemeal pine nut flour digestates obtained during gastric digestion
PILD: Wholemeal pine nut flour digestates obtained during intestinal digestion
PL3K: Phosphoinositide 3-kinase
PPI: Pea protein isolate
PPPI: *Pinus pinea* L. protein isolate
P/T H: Pepsin/trypsin hydrolystae
P/T/A H: Pepsin/trypsin/alcalde hydrolysate
PVDF: Polyvinylidene difluoride

$\mathcal{R}$

R^2: Determination coefficient
RGE: Rabbit gastric extract
ROS: Reactive oxygen species
RSM: Response surface methodology

$\mathcal{S}$

SDS: Sodium dodecyl sulfate polyacrylamide
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel
SGF: Simulated gastric fluid
SIF: Simulated intestinal fluid
SOL: Solubility
SPI: Soy protein isolate

$\mathcal{T}$

T/A H: Trypsin/alcalde hydrolysate
TBST: Tris-buffered saline with Tween-20
TCA: Trichloroacetic acid
TE: Trolox equivalent
TH: Trypsin hydrolysate
THD: Trypsin hydrolysate digestates obtained during intestinal digestion
THG: Trypsin hydrolysate digestates obtained during gastric digestion
TMB: tetramethylbenzidine
TNBS: Trinitrobenzenesulfonic acid

$\mathcal{U}$

U: Units

$\mathcal{V}$

v/v: volume per volume

$\mathcal{W}$

WAC: Water absorption capacity
WHC: Water holding capacity
WHO: World health organization

Amino acids list

A (Ala): Alanine

C (Cys): Cysteine

D (Asp): Aspartic Acid

E (Glu): Glutamic Acid

F (Phe): Phenylalanine

G (Gly): Glycine

H (His): Histidine

I (Ile): Isoleucine

K (Lys): Lysine

L (Leu): Leucine

M (Met): Methionine

N (Asn): Asparagine

P (Pro): Proline

Q (Gin): Glutamine

R (Arg): Arginine

S (Ser): Serine

T (Thr): Threonine

V (Val): Valine

W (Trp): Tryptophane

Y (Tyr): Tyrosine

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General introduction

General introduction

In recent years, plant-based proteins have gained prominence as favorable alternatives to animal-derived proteins across various applications, encompassing human nutrition and products promoting health (Moure et al., 2006; Sandberg, 2011; Valdés et al., 2016; Gao et al., 2018; Dapčević-Hadnađev et al., 2019; Vinayashree and Vasu, 2021).This shift is primarily attributed to the widespread availability, diversity, and lower risk of contamination associated with plant-based sources, combined with the increasing interest in natural and health-conscious dietary choices. Although plant-based proteins lack one or two essential amino acids in comparison to animal-based proteins, this deficiency can be easily addressed through supplementation with other protein sources (Moure et al., 2006; Vinayashree and Vasu, 2021). Notably, soy and pea proteins are the predominant plant-based proteins used in food applications, yet there is growing interest in exploring other plant sources like pulses, oilseeds, nuts, and grains (Ismail et al., 2020).

The use of plant-based proteins in food products aims to enhance nutritional quality and impart favorable sensory, organoleptic, and physicochemical attributes (Moure et al., 2006; Shevkani et al., 2015). These proteins are commonly represented in the forms of protein concentrates, protein isolates, and protein hydrolysates (Rodrigues et al., 2012). Proteins possess unique surface properties due to their amphiphilic nature, enabling them to offer a range of functional properties including solubility, emulsification, foaming, gelling abilities, and water/oil retention capacities (Ragab et al., 2004; Dapčević-Hadnađev et al., 2019). Incorporating plant proteins into food products is heavily reliant on their functional properties. To succeed in food applications, proteins should exhibit favorable functional traits while supplying essential amino acids (Cui et al., 2020; Mohan and Mellem, 2020c).

The most prevalent technique for protein isolation in the food industry is alkaline extraction and isoelectric precipitation (Cui et al., 2020; Gerzhova et al., 2016; Sethi et al., 2021). The structural and functional properties, yield, and purity of the extracted proteins are influenced by extraction conditions such as pH, temperature, extraction time, and liquid-to-solid ratio (Cai et al., 2020; Gao et al., 2018; L'hocine, Boye, & Arcand, 2006; Sethi et al., 2021). The pH parameter during alkaline treatment is particularly influential, as it can alter protein conformation, unfolding, and charge, leading to significant changes in functional properties (Cui et al., 2020; López et al., 2018; Valenzuela et al., 2013).

Pinus pinea L. pine nuts are prominent edible seeds cultivated mainly in the Mediterranean

region and valued globally for their nutrition and taste (Evaristo et al., 2013; Loewe-Muñoz et al., 2018; Zuleta et al., 2018). Despite being energy-dense and high in fat, they are incorporated in the Mediterranean diet for flavor and are associated with various health benefits, including reduced cardiovascular risk and improved body index. With significant fat (19.8-44.9%), protein (13-31.9%), and carbohydrate (1.16-5.15%) content, along with essential vitamins, pine nuts are highly nutritious (Nergiz and Dönmez, 2004; Kadri et al., 2015; Zuleta et al., 2018). Although pine nuts are often studied for their oil content, their protein content has been underexplored. Given their substantial protein content, pine nuts offer potential as a valuable plant protein source for the food industry and the development of new functional ingredients.

Reactive oxygen species (ROS) are generated as byproducts during various oxidative physiological and biochemical processes. Under normal physiological circumstances, the body's defense system against oxidation employs both enzymes (e.g., superoxide dismutase, catalase, glutathione peroxidase) and non-enzyme antioxidants (e.g., melatonin, glutathione, coenzymes, cofactors, tocopherol, ascorbic acid) to counteract the harmful impacts of these molecules (Chen et al., 2016). Nonetheless, an imbalance between free radicals and the body's intrinsic antioxidant defense can result in oxidative stress within cells, causing damage to various cellular components such as DNA, proteins, and membrane lipids. This oxidative damage is linked to the development of chronic diseases such as diabetes, Alzheimer's disease, cardiovascular diseases, and cancer (Rice-Evans and Diplock, 1993).

There is a growing consumer desire for protein with both high nutritional and functional attributes. This has led researchers to shift their focus from conventional protein sources towards plant-based proteins. Notably, plant-derived protein hydrolysates and their isolated bioactive peptides have gained global prominence due to their advantages in terms of sustainability, cost-effectiveness, environmental preservation, and lack of harmful side effects (Wen et al., 2020).

The enzymatic hydrolysis process, employing proteases from various sources, is extensively utilized to produce protein hydrolysates from food sources that possess antioxidant properties (Mullally et al., 1994). The antioxidant efficacy of these protein hydrolysates and bioactive peptides has predominantly been assessed through *in vitro* models. These models rely on different mechanisms, including free radical scavenging, reduction potential, and iron chelation abilities (Carocho et al., 2018; Kumar et al., 2019). However, for bioactive peptides to exert physiological effects *in vivo*, they must withstand digestion in the gastrointestinal tract and reach

their target sites in an active form following absorption (Wu et al., 2015; Espejo-Carpio et al., 2016). Additionally, gastrointestinal digestion, recognized as a primary site of oxidation within the human body, can lead to modifications in the functional capabilities of bioactive peptides (Srigiridhar et al., 2001). As a result, evaluating the stability of bioactive peptides post-digestion is vital. The use of an *in vitro* simulated gastrointestinal digestion model is a widely accepted method to obtain preliminary insights into the peptides' bioavailability before conducting *in vivo* investigations (Samaranayaka et al., 2010; Teixeira et al., 2016).

The main aim of this thesis is to comprehensively study and valorize pine nuts (*Pinus pinea* L.) protein isolates and hydrolysates through a variety of approaches. To achieve this aim, response surface methodology was utilized to optimize alkali extraction and isoelectric precipitation conditions, aimed at understanding their influence on the functional properties and purity of the protein isolates. The subsequent section of the study focused on generating protein hydrolysates through enzymatic hydrolysis, employing pepsin, trypsin, and Alcalase enzymes. The antioxidant activity of the generated hydrolysates was evaluated using multiple *in vitro* tests, including DPPH, ABTS, and OH radical scavenging activities, iron chelation activity, ferric reducing antioxidant power, and ORAC assay. Lastly, the bioavailability of trypsin hydrolysate was assessed using simulated *in vitro* gastrointestinal digestion. Moreover, the potential impact of enzymatic hydrolysis on the allergenicity of the protein isolate and hydrolysate was also examined.

For presentation purposes, this thesis is structured into three sections. **Part I** encompasses the literature review, which is composed of two chapters. **Chapter I** delves into plant-based proteins and offers an overview of *Pinus pinea* L. nuts. **Chapter II** provides general insights into protein hydrolysates and bioactive peptides, along with their associated biological activities.

After establishing this foundation through literature review, **Part II** outlines the research questions, hypotheses, and objectives that drive this study.

Finally, **Part III** represents the core research conducted within this thesis and is presented across three chapters, each encompassing materials and methods, results, and discussion.

Part I. Literature review

Chapter I. Plant-based proteins

Chapter I. Plant-based proteins

I.1. General information on proteins

The term "protein" was first introduced into the scientific literature in 1838 by Gerardus Mulder, a Dutch chemist, in his publication titled "On the composition of some animal substances", where he described the presence of a common complex substance in blood fibrin, serum, egg albumin, gelatin, and wheat gluten (Mulder, 1839). The word "protein" originates from the Greek word *Proteios*, which means of first rank or first position, and aptly reflects the vital role proteins play in living organisms (Yao et al., 2020). Proteins are the most important macromolecules found in living cells, they make up more than half (50%) of a cell's dry weight and approximately 20% of the body's total mass. They perform vital functions in the body as enzyme catalysts, hormones, regulators, transporters, and structural molecules involved in key immune, circulatory, and homeostatic processes (Cozzone, 2002; Murray et al., 2017).

Proteins are biopolymers made up essentially of amino acids linked together by peptide bonds formed between the primary carboxyl and amine groups (Aluko, 2015). In nature, there are 20 standard amino acids that are commonly found (McClements and Grossmann, 2022). The canonical structure of amino acids consists of an alpha carbon (C_α) covalently bonded to three different components - a hydrogen, a carboxylic carbon, an amino nitrogen, and an amino acid-specific side chain (R) (Murray et al., 2017) (figure 1). The side chains of the amino acids contribute to the protein's characteristics, structure, and function (Murray et al., 2017; Balandrán-Quintana et al., 2019).

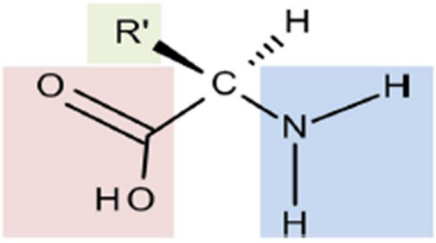

Figure 1: General structure of amino acids (Murray et al., 2017).

I.1.1. Protein structure

Proteins consist of a long chain of amino acid residues that fold into distinctive structures. These folds include one or more specific spatial conformations determined by a number of covalent and noncovalent interactions. These interactions include hydrogen bonding, ionic

interactions, Van der Waals forces, hydrophobic packing, salt bridges, disulfide bonding, and post-translational modifications (Aryee et al., 2018). The significance of these interactions depends on the protein's primary structure, as well as the environmental conditions such as pH, ionic strength, and temperature (McClements and Grossmann, 2022). In terms of structure, proteins are categorized into four hierarchical levels: primary, secondary, tertiary, and quaternary (Rodrigues et al., 2012; Murray et al., 2017) **(**Figure 2).

I.1.1.1. Primary structure

The primary structure refers to the linear order in which amino acid residues are arranged along the polypeptide chain. This arrangement arises from the covalent linkage of individual amino acids via peptide bonds. Each protein possesses a unique sequence of residues, and all subsequent levels of organization (secondary, super secondary, tertiary and quaternary) are based on this primary level of structure (Whitford, 2013). The primary structure of proteins is governed by the DNA of the organism that produced them. Proteins differ in their primary structure because they are designed, under evolutionary pressure, to perform different functions in nature. The number, type, and sequence of amino acids in the polypeptide chain ultimately determine their conformation in the present natural environment (McClements and Grossmann, 2022).

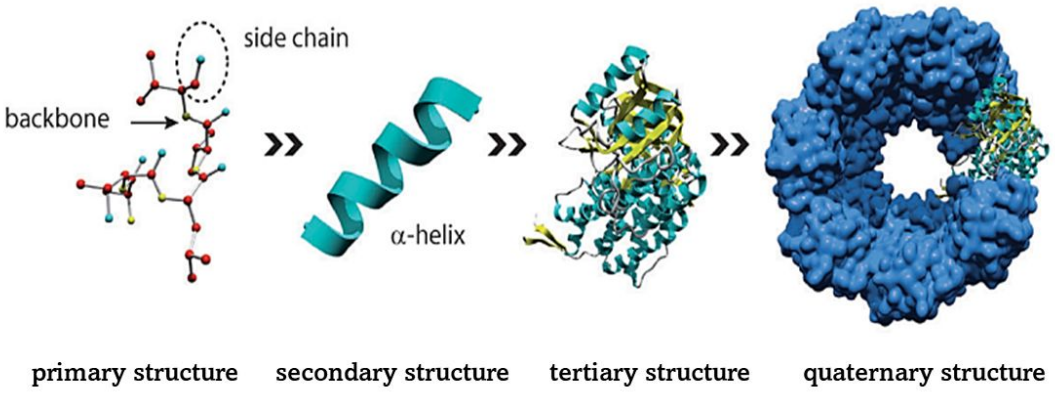

Figure 2: The hierarchical structure of proteins (primary, secondary, tertiary, and quaternary) (Heim et al., 2010).

I.1.1.2. Secondary structure

The Secondary structure refers to the presence of local regions within the polypeptide chain

that exhibit certain structural organizations (McClements and Grossmann, 2022). The most common secondary structure is the α-helix, followed by the β-sheet. Other secondary structures include the reverse turns (Murray et al., 2017).

I.1.1.2.1. α-helix

The α-helix is formed through interactions within the same strand, where the carbonyl group of each peptide residue in the helix forms hydrogen bonds with the amide group of the peptide located four residues away in the sequence. The helix, which is typically right-handed, consists of 3.6 residues per turn, with a rise of 1.5 A˚ per residue, resulting in a pitch of 5.4 A˚. While any amino acid can be found in an α-helix, different amino acids have varying tendencies to adopt this conformation. This propensity is known as *helix propensity*, with proline and glycine having the lowest propensity of appearing in a helix (Pauling et al., 1951; Pace and Scholtz, 1998; Murray et al., 2017).

I.1.1.2.2. β-Sheet

The β-sheet is the second most prevalent secondary structure and occurs when segments of a polypeptide chain, known as β-strands, overlap, and form hydrogen bonds between the strands. Similar to the α-helix, the stability of the β-pleated sheet is attributed to its hydrogen bonds as well as to the Van der Waals forces resulting from the close proximity of the residues within the structure. The β strands within the β-sheet structure are in an almost fully extended conformation and can run parallel or antiparallel, following the conventional N-terminus to C-terminus directionality (Murray et al., 2017).

I.1.1.2.3. Reverse Turns

This characteristic represents another commonly observed secondary structure in proteins, where the polypeptide chain folds back on itself at an approximate angle of 180 degrees, resulting in a change in chain direction. The most common type of reverse turn is the β-turn, which consists of four residues. In the β-turn, residue i and i+3 form hydrogen bonds, while residues i+1 and i+2 play a role in the actual bending of the polypeptide chain. The interaction between the carbonyl group and amide nitrogen of residues i and i+3 further stabilizes the structure. If the β-turn is flanked by two β-strands, the structure is referred to as a β-hairpin. Reverse turns are a common type in globular proteins since the folding of the polypeptide chain is crucial for the formation of their compact three-dimensional (3D) structures (Murray et al., 2017).

I.1.1.3. Tertiary structure

The tertiary structure represents the three-dimensional shape that a protein adopts when it folds upon itself. This folding is stabilized by weak interactions between polar and nonpolar groups. At the tertiary level, the secondary structure elements come together, packed tightly to form a cohesive tertiary object. Although there are instances where tight turns connect secondary structure elements together like α-helices and β-strands, more commonly, there are long stretches of amino acids that lack a regular structure between these secondary elements. These stretches are often located on the protein's surface, exposed to the surrounding solvent. As a result, they serve as convenient sites for recognition, interaction, and binding sites, making them crucial for protein function (Murray et al., 2017).

I.1.1.4. Quaternary structure

The quaternary structure of proteins refers to their larger-scale arrangement, where they form supramolecular structures through physical and/or chemical bonds. Each unit of the tertiary structure is called a subunit within this arrangement. Quaternary structures can be categorized as homo-oligomers (with identical subunits) or hetero-oligomers (with a mixture of different subunits). Homodimers, consisting of two identical subunits, represent the simplest form of quaternary structures. One advantage of quaternary structures is their ability to facilitate efficient repairs by easily replacing defective subunits. The subunits within these structures interact through complementary regions on their surfaces, and studies suggest that the flexibility of subunits influences their assembly (Murray et al., 2017; McClements and Grossmann, 2022).

The conformation adopted by a protein in its natural environment is referred to as the *native state*, which typically corresponds to the configuration of the polypeptide chain that gives the lowest free energy. The native structure of a protein is crucial for its biological functions, including enzyme activity, signaling, transport, motility, mechanical properties, and structure formation. When proteins are isolated from their natural environment, changes may occur in their tertiary and quaternary structures due to shifts in molecular interactions. Additionally, the attainment of the lowest free energy state by polypeptide chains in a specific environment can be hindered by kinetic energy barriers. Consequently, a protein may become trapped in one or more *denatured states* because it cannot overcome these kinetic energy barriers and reach its native state (McClements and Grossmann, 2022).

I.1.2. Classification of proteins

Proteins can be classified based on various properties. One classification is based on their chemical composition, dividing proteins into two sub-categories: homoproteins, which are composed solely of amino acids (e.g., protamines, histones, glutenins, albumins, prolamins, globulins, and scleroproteins); and heteroproteins, which consist of amino acids along with nonprotein components known as prosthetic groups (e.g., glycoproteins, phosphoproteins, lipoproteins, metalloproteins, and nucleoproteins) (Rodrigues et al., 2012). Another classification of proteins is based on their shape, distinguishing them as globular, flexible, or fibrous. Globular proteins have compact and spherical structures. Flexible proteins exhibit relatively disordered structures with a high level of conformational mobility. Fibrous proteins, on the other hand, are generally stiff and extended, often formed by polypeptide chains adopting helical structures (McClements and Grossmann, 2022) (figure 3).

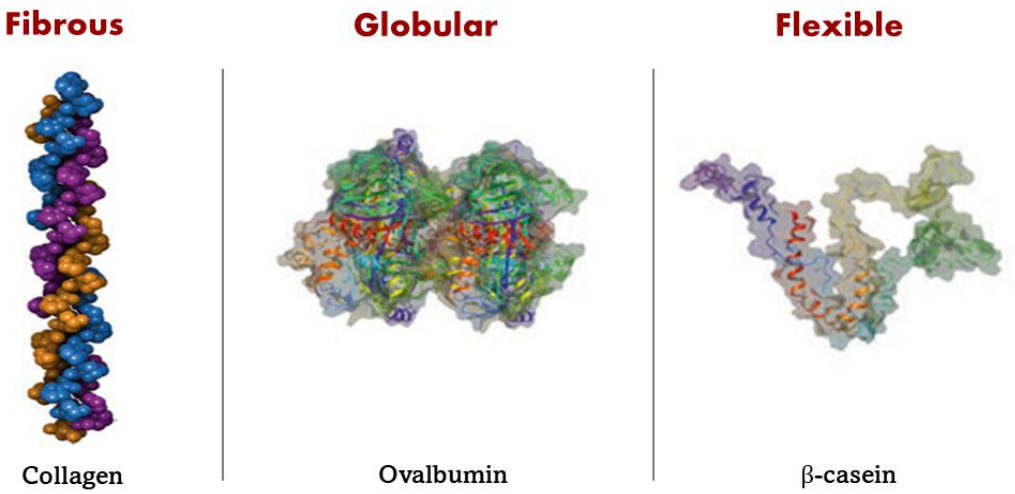

Figure 3: The different tertiary structure of proteins in foods, globular (e.g., ovalbumin), flexible (e.g., β -casein), or fibrous (e.g., ovalbumin) depending on the conformations of their polypeptide chains (McClements and Grossmann, 2022).

I.2. Plant-based proteins

Plant-based proteins, as the name suggests, are proteins derived from plants. They play a crucial role in vegetarian or vegan diets and are favored by those aiming to decrease their consumption of animal products (Rizzo and Baroni, 2018). Examples of plant-based protein sources include legumes (such as soybeans, peas, beans, chickpeas, lupins, faba beans, and

cowpeas) (Lqari et al., 2002; Coda et al., 2017; Sá et al., 2020), cereals (like rice, wheat, millet, sorghum, maize, and barley), pseudocereals (including amaranth, quinoa, and buckwheat) (López et al., 2018a), seeds (such as chia, flaxseed, sesame, pumpkin, and sunflower), as well as almonds and nuts (Conde et al., 2005; de Oliveira Sousa et al., 2011; Mattila et al., 2018).

In recent times, plant proteins have gained popularity as a cost-effective and versatile alternative to animal sources in human nutrition. They are also utilized as functional ingredients in product development. The use of animal protein comes with increasing costs, limited supply, and various negative impacts on the environment and human health, including climate change, water depletion, biodiversity loss, and risks associated with cardiovascular diseases. Consequently, there is a global trend shifting from animal proteins to plant-based proteins while still meeting nutritional requirements, particularly in western countries (Sá et al., 2020; Qin et al., 2022).

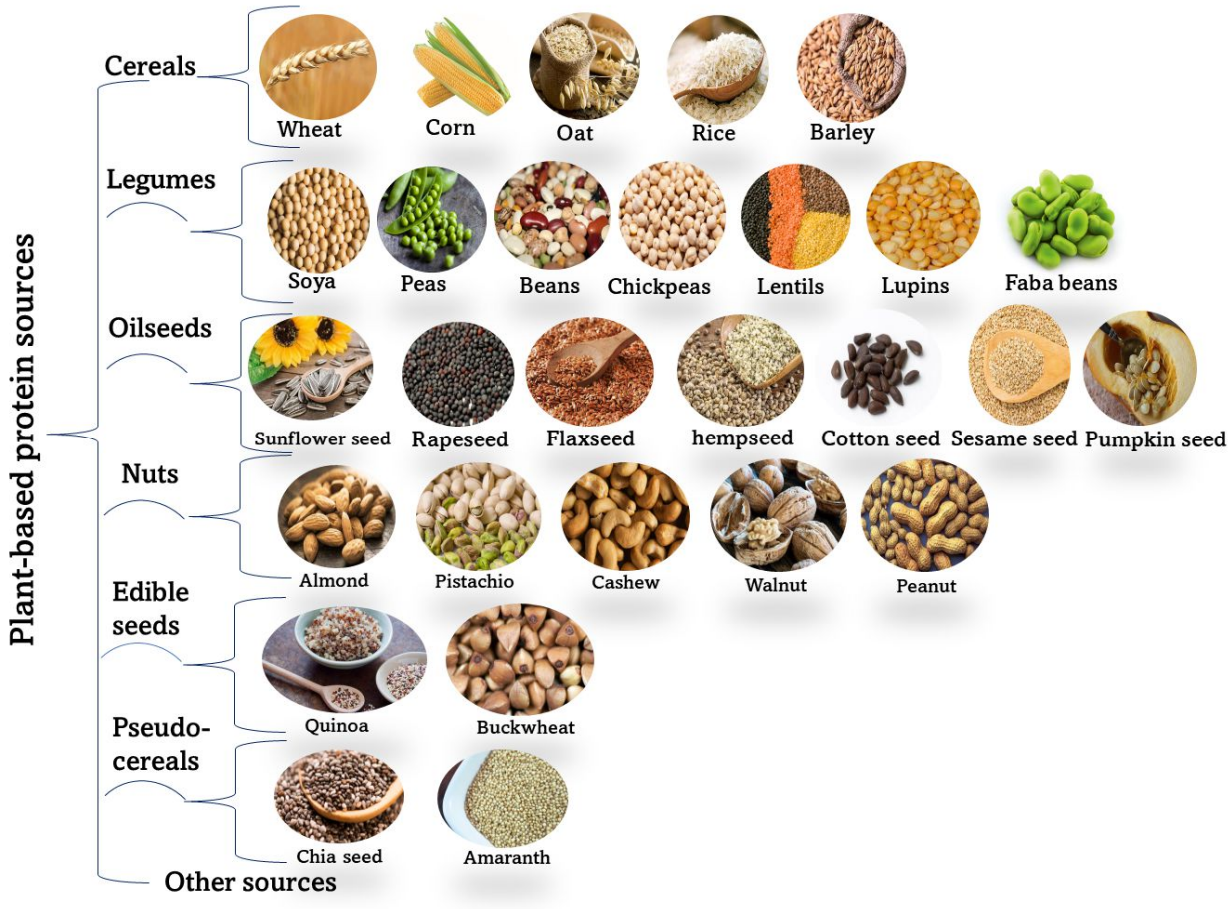

Figure 4: Major plant-based protein sources.

Plant-based proteins offer a promising solution due to their extensive history of cultivation

and use in agriculture, lower production costs, and widespread availability in many regions. They contribute to reducing cholesterol levels, promoting bone health, enhancing muscle mass in older individuals, meeting the protein needs of athletes, and are generally more environmentally sustainable (Jenkins et al., 2010; Willett et al., 2019; Sim et al., 2021; Kumar et al., 2022).

Plant proteins consist primarily of globular proteins, which exist as multimers connected by covalent bonds. They can be categorized into four groups based on their solubility properties: albumins (soluble in water), globulins (soluble in weak salt solutions), prolamins (soluble in ethanol-water mixtures), and glutelins (soluble in dilute acid/alkaline solutions or insoluble in water) (Grossmann and Weiss, 2021). Albumins and globulins are the predominant protein types found in most legumes (>50%) and some pseudocereals like quinoa and amaranth (Boye et al., 2010b). On the other hand, prolamins (found in wheat, maize, barley, and rye) and glutelins (found in wheat) make up around 85% of the total protein content in cereals and pseudocereals (Veraverbeke and Delcour, 2002; Delcour et al., 2012; Grossmann and Weiss, 2021). These globular proteins consist of polypeptide chains that fold tightly together due to various forces such as hydrophobic effects, hydrogen bonding, electrostatic forces, van der Waals forces, and disulfide bonds. Prolamins and glutelins exhibit similarities in terms of the proportion of proline and glutamine amino acids, as well as their amino acid sequences. However, they differ in terms of their molecular weight, as well as their intra- and intermolecular structures (González-Pérez and Arellano, 2009).

I.2.1. Nutritional quality of plant-based proteins

Proteins are crucial macronutrients that play a vital role in human nutrition and overall well-being. Evaluating the nutritional quality of proteins involves considering their essential amino acid profile, digestibility, and bioavailability. Several methods are employed to assess this quality. The PDCAAS, or protein digestibility-corrected amino acid score, is used to determine the protein's quality by measuring its ability to meet the amino acid requirements of the human body. The biological value (BV) indicates the proportion of absorbed amino acids from a food source that is utilized to form proteins in the body (Kumar et al., 2022; Qin et al., 2022). Net protein utilization (NPU) compares the mass of amino acids transformed into protein with the mass of amino acids absorbed, providing insight into how efficiently the protein is utilized body. Plant-based proteins generally exhibit lower PDCAAS, BV, and NPU compared to animal proteins. This is primarily because plant proteins have different structural characteristics, such as an abundance

of β-sheet structures and fewer α-helices, making them more resistant to enzymatic digestion. Additionally, the presence of dietary fiber and anti-nutritional factors in plant proteins further hampers their digestibility. However, through various processing techniques, these factors can be reduced, thereby improving the digestibility of plant proteins (Kumar et al., 2022).

Amino acids are the primary components of proteins in the human body and are essential for their structure and function Each amino acid has a unique and important role in the functioning of organisms (Qin et al., 2022). Some amino acids, known as essential amino acids, cannot be produced by the human body and must be obtained through diet. These essential amino acids include histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Sá et al., 2020).

Proteins derived from plant sources may lack certain essential amino acids, making them incomplete protein sources. However, exceptions exist, such as soy proteins, which offer a high-quality protein profile similar to that of milk and whey (Qin et al., 2022). Cereal proteins generally have low levels of lysine, while legume proteins lack sulfur-containing amino acids like methionine and cysteine. Nevertheless, pseudo-cereals like amaranth and quinoa are good sources of lysine (Sá et al., 2020). Due to these variations in amino acid content, relying on a single plant protein source in a formulated product is insufficient to provide all the necessary amino acids for a balanced diet. It is important to combine different plant foods, such as cereals and pulses, to ensure an adequate intake of essential amino acids and promote vascular health (de Boer et al., 2006; Martínez-González et al., 2017; Ahnen et al., 2019).

I.2.2. Plant-based protein ingredients

Plant protein ingredients are available in different forms, including flours, concentrates, and isolates, which are determined by their protein concentrations. The concentrations typically fall within the ranges of 50% to 70%, over 80%, and over 90% for these respective forms. The protein content increases as more extensive and expensive extraction processes are employed (McClements and Grossmann, 2022).

Protein ingredients comprise various types of proteins, each exhibiting different molecular conformations (native or denatured) and aggregation states (such as monomers, dimers, and trimers). These characteristics are influenced by factors like the biological origin of the proteins and the specific extraction and drying methods used. The concentration, type, conformation, and aggregation state of proteins within an ingredient significantly affects its functionality, playing a

vital role in determining its performance in different applications. Additionally, protein ingredients contain non-protein components like starches, fibers, lipids, and minerals, which can impact their functional performance (Asgar et al., 2010; Ma et al., 2022).

It is believed that protein ingredients display diverse functional properties, and this variation in performance among plant proteins is strongly influenced by protein concentration and the specific form of the ingredient utilized. Therefore, it is essential to identify a protein ingredient that possesses the desired molecular, physicochemical, and functional attributes required for a specific application (Ma et al., 2022).

I.2.3. Functional properties

Proteins are surface active molecules, they possess the ability to form monolayers on aqueous solutions, as well as interlayers between oil and water. Additionally, they can come together to create clusters and micelles within the bulk, forming extensive supramolecular structures that can gelate the water phase. Moreover, proteins have the capability to form emulsions that exhibit gel-like characteristics (Hoffmann and Reger, 2014).

Functional properties of proteins refer to how proteins behave and interact within the food system. These properties are influenced by the physical, chemical, and conformational characteristics of proteins during various stages such as processing, storage, cooking, and consumption. The functional properties are determined by factors like molecular weight, size, shape, flexibility, amino acid composition, structure, net charge, charge distribution, and hydrophobicity. The functionality of proteins can vary depending on the protein source, composition, preparation method, thermal history, and environmental factors such as pH, ionic strength, temperature, and presence of salts. The relationship between protein structure and functional properties is significant in food systems, and understanding this correlation is crucial for effectively utilizing proteins in food applications (Phillips and Williams, 2009).

I.2.3.1. Solubility

The solubility of proteins refers to the amount of nitrogen in a protein product that can dissolve under specific conditions. When proteins are used as food additives, they can be partially or fully soluble in water, or completely insoluble. Assessing protein solubility is typically the initial step in evaluating new protein ingredients, as it is connected to other functional properties. Understanding protein solubility provides valuable insights into the potential applications of

proteins and their effectiveness, particularly in creating foams, emulsions, and gels (Zayas and Zayas, 1997).

Proteins chosen for inclusion in liquid foods and beverages are primarily selected based on their solubility, which is their primary attribute. Proteins with high solubility exhibit excellent dispersibility of their molecules or particles, resulting in the creation of finely dispersed colloidal systems. If proteins have high solubility, their potential applications can be significantly broadened, opening up more possibilities for their use (Zayas and Zayas, 1997).

The characteristics of proteins in a solution can be affected by their surface properties, specifically the presence and arrangement of hydrophilic and hydrophobic amino acid groups on the surface. In water, hydrophilic amino acids have a tendency to face the surrounding solvent, while hydrophobic residues are typically concealed within the protein structure to minimize energy expenditure. Protein solubility can also be influenced by factors such as the composition and sequence of amino acids, molecular weight, and conformation. Environmental conditions like ionic strength, solvent composition, pH, temperature, and processing conditions can also impact protein solubility (Hall, 1996; Zayas and Zayas, 1997; Damodaran et al., 2007).

I.2.3.2. Water-holding capacity

The water-holding capacity of proteins refers to their ability to retain water against the force of gravity (Shevkani et al., 2015). Water binding occurs through various interactions, including ion-dipole, dipole-dipole, dipole-induced dipole, and hydrophobic interactions (Damodaran et al., 2007). Several models have been suggested to explain how water binds to proteins. Typically, water that is bound to proteins is closely associated with them and cannot freeze or serve as a solvent for chemical reactions (Hall, 1996). On the other hand, retained or immobilized water is trapped within the protein structure and can be expelled through external forces like centrifugation or pressing (Stone et al., 2015).

The water holding capacity of a protein is influenced by its amino acid composition. Water molecules attach to different groups within amino acids, such as charged groups, backbone peptide groups, amide groups, hydroxyl groups, and nonpolar residues, each with varying abilities to bind water. Proteins with a high charge content demonstrate stronger electrostatic attraction towards water. Additionally, the water holding capacity is at its minimum when the protein's pH is at its isoelectric point, as this is when protein-protein interactions are most pronounced (Damodaran et

al., 2007; Stone et al., 2015).

I.2.3.3. Oil-holding capacity

Oil-holding capacity refers to the quantity of oil that can be absorbed per gram of protein (Lin and Zayas, 1987). The interaction between lipids and proteins occurs through the binding of lipid aliphatic chains to the nonpolar side chains of amino acids. Consequently, proteins with higher hydrophobicity tend to possess a greater ability to retain oils (Sanjeewa, 2008; Withana-Gamage et al., 2011). The values of oil-holding capacity can be influenced by the protein's matrix structure, the specific type of lipid involved, and the distribution and stability of lipids. These factors are affected by the size and distribution of lipid droplets as well as the presence of emulsifying agents (Hall, 1996). Understanding the oil-holding capacity is crucial as it correlates with the protein's emulsifying ability (Boye et al., 2010a).

I.2.3.4. Emulsifying properties

An emulsion is formed by mechanically agitating two liquids that do not mix, resulting in tiny droplets of one liquid dispersed within the other liquid. This creates a dispersed phase of submicron droplets suspended in a continuous phase (Hall, 1996). In the context of food, emulsions can be of the oil-in-water type (like milk and mayonnaise) or the water-in-oil type (like butter and margarine) (Alzagtat and Alli, 2002). Emulsions are thermodynamically unstable because this arrangement increases the interfacial area and, consequently, the interfacial free energy of the system. As a result, oil-in-water emulsions are susceptible to creaming, flocculation, and coalescence over time, as the system tries to minimize its free energy (Walstra, 2003).

Proteins adsorb to the interface between the two liquid phases to reduce the interfacial tension (Alzagtat and Alli, 2002). They align themselves at the interface based on their amphiphilic nature, adopting configurations such as trains, loops, and tails to form a viscoelastic film. Trains are located along the interface, while loops and tails extend into the continuous phase to facilitate repulsion. The protein's net charge and its ability to quickly reorient at the interface determine its molecular flexibility, which is considered the most crucial characteristic of a good emulsifier (Damodaran et al., 2007).

I.2.3.5. Foaming properties

The foaming capacity of a protein refers to its ability to create interfacial area in a foam. This capacity is positively correlated with the protein's average hydrophobicity and can be improved by partially denaturing the protein to increase its surface activity. Foams are dispersions of gas bubbles within a liquid (typically water) or a solid continuous phase. They can be generated through methods such as sparging, whipping, shaking, or pouring. Foams are thermodynamically unstable due to the high free energy at the gas-liquid interface, leading to coalescence and disproportionation to reduce the interfacial area (Hall, 1996; Dickinson, 2010).

Solubilized proteins diffuse and adsorb to the gas-liquid interface, reducing surface tension. They unfold and orient their hydrophobic regions towards the gas phase and hydrophilic regions towards the liquid phase, forming train and loop structures. The interactions between polypeptides result in the formation of a cohesive, continuous film around the gas bubbles (Kinsella, 1981; Wierenga and Gruppen, 2010).

Foam stability refers to a protein's ability to withstand various stresses and maintain the foam structure. Stable foams exhibit resistance to gas diffusion, drainage, thinning of the fluid film, and mechanical shock. To achieve stability, protein-based foams should have cohesive interfacial films that are maintained through hydrogen bonding, electrostatic interactions, and hydrophobic interactions (Damodaran et al., 2007; Lam et al., 2018).

I.2.3.6. Gelation

A protein gel is a well-defined three-dimensional network formed by protein molecules immersed in an aqueous solvent (Lam et al., 2018). Gelation is a crucial functional property of globular proteins as it enables the modification of food texture (Ikeda and Nishinari, 2001). The gelation process of globular proteins involves two stages: a conformational change or partial denaturation of the protein molecules, followed by their gradual association or aggregation to create a three-dimensional matrix structure that entraps water, fat, and other food components (Gaonkar and McPherson, 2016). Protein gelation can be induced by various factors such as heat treatment, pH, salts, pressure, shearing, and the presence of different solvents (Yh and Hui, 2006). The primary method of forming food protein gels is typically through heat treatment (Lam et al., 2018).

Table I: Functional properties of some plant-derived proteins.

plant source	extraction technique	functional properties studied	values	concluding remarks	reference
			Nuts		
Almond	Aqueous and enzyme-assisted aqueous extraction	SOL (pH 5) WAC OAC EC FC (pH 2)	47-23% 2.71-2.42 g g^{-1} 3.1-2.9 g g^{-1} 492-402 g g^{-1} 91.7-65%	Proteins showed strong WAC and OAC, and proteolysis enhanced solubility at pH 4–5, improving emulsification and foaming	(Dias and de Moura Bell, 2022)
Wild almond	Alkaline extraction and isoelectric precipitation (AE/IP)	WAC OAC SOL EA ES FC (pH 2) FS (pH 2)	2.3-2.6 g g^{-1} 3.1-3.5 g g^{-1} 85.3-85.8% 35-28 m^2/g 33-28 min 42.3-34.5% 70-100%	High solubility in alkaline conditions, acceptable Fc values, with significant FS at extreme pH	(Amirshaghaghi et al., 2017)
Cashew nut	Aqueous-isoelectric extraction method	WAC OAC EA ES FC FS	1.74 mL/g 3.32 mL/g 13.7% 153% 40% 40%	Protein isolate exhibits superior water/oil absorption, emulsifying stability, and foam properties	(Ogunwolu et al., 2009)
Walnut	AE/IP + ultrasound (US) treatment	SOL EC ES	76.4-93.2% 35.4-44.7 m^2/g 23-32.2 min	Sonication boosted water solubility, EA, and ES	(Zhu et al., 2018)
Pecan	US-assisted enzymatic extraction	SOL EA ES	70.77% 120 m^2/g 202 min	Enhanced functional properties were acquired with US-assisted enzymatic method	(Wang et al., 2021)
	US-assisted extraction	SOL EA ES	40.43% 97.62 m^2/g 424 min		
	Enzymatic extraction	SOL EA ES	47.98% 82.87 m^2/g 120 min		
Hazelnut	AE/IP	WAC OAC FC FS (pH 9.5) EC ES	1.16-4.10 g g^{-1} 2.07-4.02 mL/g 51-54 mL 6-7% 20.45-29.12% 31.90-50.35%	Purification process enhanced protein functionality, with higher solubility at alkaline pH than acidic	(Tatar et al., 2015)
Pistachio	Defatting	OAC WAC EC ES	3.10 mL/g 2.25 mL/g 25.2% 15.9%	Processing (heating and autoclave) impacts functional properties	(Sanchiz et al., 2019)

		FC FS	47.4% 45.7%		
peanut	AE/IP + Enzymatic hydrolysis	SOL (pH>7) EA (pH 7)	80% 22.2 m^2/g	Enzymatic hydrolysis enhances protein isolate's functionality	(Zhao et al., 2011)
			Oilseeds		
Rapeseed	AE	SOL WHC OAC EC (pH 5) ES	65% 3.85 mL/g 4.20 mL/g 373 mL/g 60%	Steaming decreased OAC, EC, and solubility. Adding steamed rapeseed protein to sausages improved taste, texture, and aroma in sensory analysis compared to casein	(Yoshie-Stark et al., 2006)
flaxseed	AE/IP	SOL (pH7) WAC OAC EA ES	57% ~2.8 g g^{-1} ~4 g g^{-1} 375 m^2/g 179.5 h	Low solubility, but superior emulsion properties compared to common proteins	(Kaushik et al., 2016)
Sunflower	AE/IP + heat treatment	SOL (pH 5.5, 25 min) EA ES FC FS WAC (pH5.5) OAC (pH5.5)	51% 49.56% 55.52% 50.25% 55.58% 149% ~ 95%	Heat treatment induces protein denaturation, causing alterations in protein structure and subsequently enhancing functional properties	(Malik and Saini, 2018)
Hempseed	AE/IP	SOL (pH7) FC FS (30min) EA ES (48h) WAC OAC	14.50-17.50% 52.9-84.9% 68.1-89.4% 1.1-3.5 m^2/g 38-73% 0.83-1.05g g^{-1} 1.28-1.81g g^{-1}	Protein functionality in Hempseed proteins is influenced by varying edestin, vicilin, and albumin ratios	(Liu et al., 2023)
			Legumes		
Soy	AE/IP + US treatment	SOL (pH 9) EC FC FS	~95% 350-450 g g^{-1} 0.71-0.98 6.11-8.3 mL/min	Intense ultrasound yields only minor changes in functional characteristics	(Karki et al., 2009).
Pea	AE/IP	SOL (pH10) WAC OAC EA ES FC FS	~100% ~180% ~80% ~48% m^2/g 15 min 20% 20 min	Pea proteins show considerable potential as meat alternatives in Frankfurt sausages and offer substantial opportunities for enrichment in bakery goods	(Tömösközi et al., 2001)
Chickpeas	AE/IP	SOL WAC OAC EA ES FC	48.33-58.3% 2.06-2.70 g g^{-1} 1.91-2.77g g^{-1} 312.5-410.5m^2/g 124.9-164.2min 41.9-58.06%	Freeze-dried samples exhibited high WAC and OAC, whereas concentrates dried through convection showed increased EAI and ESI	(Ghribi et al., 2015)

		FS	4.68-32.25%		
Edible seeds					
Quinoa	AE/IP	SOL (pH 11) WAC (pH7) OAC (pH 7) EA (pH7) ES (pH 7)	61.3-95.3% 1.46-2.76 g g^{-1} 0.94-3.19 g g^{-1} 41.46-56.63% 25.53-51.90%	Freeze-dried protein had superior functionality compared to spray- and vacuum dried proteins due to reduced denaturation	(Shen et al., 2021)
Pseudocereals					
Amaranth	AE/IP	SOL EA ES FC FS WAC OAC	49-9-77.2% 15.3-17.7 m^2/g 85-149 min 94-250% 63-75% 1.1-3.3 g g^{-1} 3.6-6.4 g g^{-1}	Protein Solubility and emulsifying activities were closely associated, and both were highly dependent on the surface charge of amaranth proteins	(Shevkani et al., 2014).

I.2.4. Industrial application of plant-based proteins

Edible applications of proteins include their use as food ingredients, supplements, constituents of edible coatings, emulsifiers in foods, bioactive peptides/hydrolysates, and hydrogels for various nutraceutical and pharmaceutical applications, including serving as carriers of functional compounds (Kumar et al., 2022). In recent years, isolated plant proteins derived from seeds have become increasingly important on the market, contributing to the functionality, texture, and improved nutritional value of various food products. Nevertheless, foods containing non-animal seed proteins are still perceived as having inferior flavour and texture (Phillips and Williams, 2009).

Table II: Required functional properties of vegetable proteins for food applications (González-Pérez and Arellano, 2009).

Food application	Property required	Protein requirements/mechanism	Main protein sources
Dairy substitutes	Solubility, color-free, tasteless, emulsifying properties, stability to heat	Hydrophilicity, molecular flexibility, interfacial adsorption	Soybean, peanuts, lupin, pea, other legumes, and oilseeds
Bakery	Viscosity, elasticity, gelation, water binding	Hydrophobicity, disulphide crosslinks, network formation, hydrogen bonding	Cereals
Low-fat bakery products, doughnuts	Fat and flavor binding	Hydrophobic bonding entrapment	Cereals
Desserts, dressings	Solubility, emulsifying/foaming properties, fat mimetic	Hydrophilicity, molecular flexibility, interfacial adsorption	Soybean, peanut, lupin, other legumes, and oilseeds

Beverages, soups, gravies	Solubility, viscosity, acid stability	Hydrophilicity, protein solvation	Soybean, pea, vegetable protein hydrolysates, fermented cereals and legumes
Fortification	High nutritional value, solubility	Digestibility, hydrophilicity absence of allergens	Amino acid-balanced vegetable protein mixtures, hydrolysates, nutritional quality improved by fermentation and germination
Infant formula	High nutritional value, solubility, emulsifying properties, stability to heat	High digestibility, full absence of allergens, hydrophilicity, interfacial adsorption	Soybean and vegetable protein hydrolysates
Meats and sausages substitutes	Texturization solubility, emulsifying properties, water binding, fat mimetic, gelation	Hydrophilicity, network formation, water entrapment and immobilization, interfacial adsorption, disulphide crosslinks	Soybean, pea, lupin, wheat
Cheese-like products	Gelation, solubility, color-free, tasteless	Hydrophilicity, proteins solvation, network entrapment and immobilization	Soybean

I.3. Pine nuts: an overview

The pine tree, belonging to the Pineaceae family and the *Pinus* genus, is the largest and most significant group of conifers, comprising over 100 species primarily found in the northern hemisphere. Among these species, *Pinus pinea* L., also known as Mediterranean stone pine, stands out as a characteristic tree in the Mediterranean forests and woodlands (Pereira et al., 2015). This medium-sized evergreen coniferous tree can reach heights of 25-30m with trunks exceeding 2m in diameter. Its crown is distinctively umbrella-shaped, large, and flat. The trunk is usually short with numerous upward angled branches that carry foliage towards the ends. The tree's bluish-green needles grow in pairs (fascicles) with an average length of 8-15cm and emit an oniony scent. As a monoecious unisexual plant, it bears numerous pollen cones crowded around the base of new shoots, each measuring 10-20mm and having a pale orange-brown color. The seed cones are ovoid-globose, initially green and 8-12cm long, but they turn reddish-brown when mature, taking three years to ripen. The stone pine's seeds are pale brown, coated with a black powder, and measure 15-20mm in length. They are heavy and have easily detachable wings, which prevents effective wind dispersal (Viñas et al., 2016).

The stone pine (*Pinus pinea* L.) is widely distributed throughout the Mediterranean basin, primarily in coastal areas, and is particularly abundant in southwestern Europe, covering an area

of more than 700,000 hectares. The main countries with significant stone pine presence are Spain (45,000 ha), Portugal (90,000 ha), Turkey (50,000 ha), and Italy (40,000 ha) (Pereira et al., 2015). Additionally, they are found in Tunisia, Algeria, Morocco, and Greece. This tree is adaptable to various climate and soil conditions, thriving in dry weather, strong direct sunlight, high temperatures, and even tolerating light-shaded conditions during its early growth stages (Viñas et al., 2016).

Pinus pinea L. holds great economic significance in the Mediterranean area, mainly due to the value of its tree seeds and cones. While the cones have been used for wood-based panels, the nuts derived from them are highly prized in the food industry (Mutke et al., 2012).

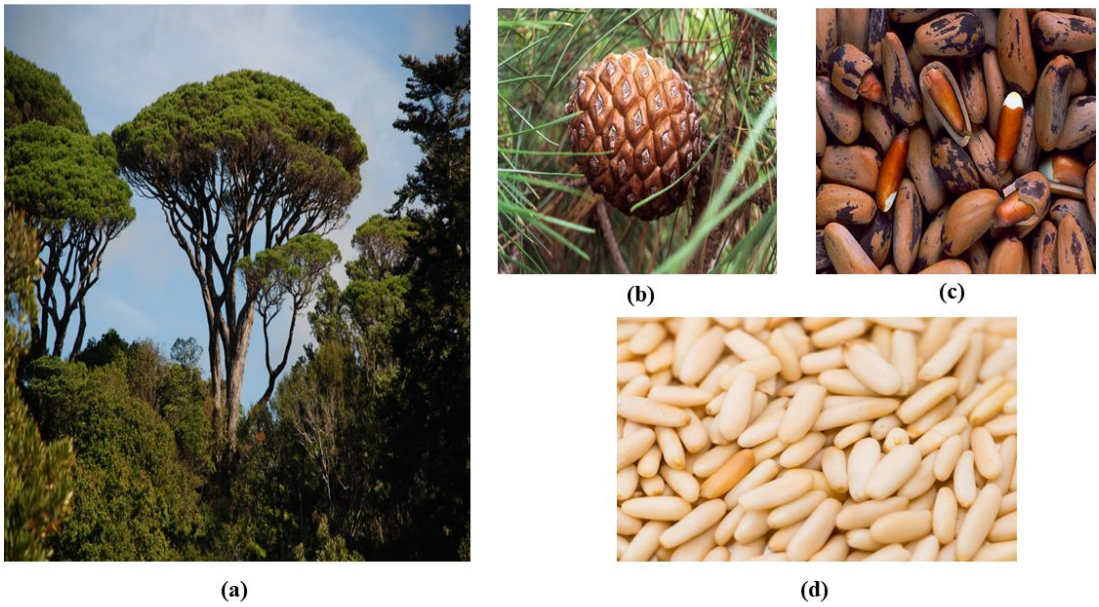

Figure 5: Stone pine (*Pinus pinea* L.) tree (a), large mature cone (b), pine nuts in their natural, unshelled and unskinned state (c), and shelled and skinned pine nuts (d).

The stone pine produces the most valuable edible wild seeds harvested from Mediterranean forests, known as pine nuts. These pine nuts are renowned worldwide for their delicate flavor, buttery taste, creamy texture, and high nutritional value (Queirós et al., 2020). They are widely recognized as a nutritious snack, both raw and roasted, and are commonly found in various nut mixes. Additionally, pine nuts are extensively used as a culinary ingredient in dishes, salads, confectionery, bakery products, and notably, in the preparation of the renowned pesto sauce. The nutritional profile of pine nuts varies depending on the species, but overall, they are rich in polyunsaturated fats, thiamin, vitamin E and K, as well as minerals such as iron, magnesium,

phosphorus, zinc, copper, and manganese. They also serve as a source of fiber, potassium, niacin, and riboflavin (Loewe-Muñoz et al., 2018; Zuleta et al., 2018).

Pine nuts can undergo pressing to yield a premium cooking oil, with the highest quality achieved through cold pressing. Pine nut oil finds application in cosmetics, and beauty products, and serves as a luxurious massage oil. The by-product of pine nut oil extraction, known as pine nut flakes, contains up to 30% oil and is commonly used in granolas, chocolates, and bars. These flakes, when further pressed and crushed, produce pine nut meal or flour, offering versatile culinary uses in pastries, pancakes, and more. When mixed with water, the meal transforms into a beverage or cream (Sharashkin and Gold, 2004).

The pine nuts of *Pinus pinea* L. are recognized as one of the world's most expensive nuts, contributing to a value chain worth several hundred million euros annually (Sharashkin and Gold, 2004). Global production of consumable pine nuts averages around 30,000 tons, with the yearly average being 27,975 tons based on 2004 data, and 19,575 tons according to 2015 data (Yalim et al., 2022). Over the past two decades, the cultivation of stone pines as nut crops has been increasing, with approximately 0.3 million hectares of new plantations established within its native range, and to a limited extent in New Zealand, Australia, and Chile (Carrasquinho et al., 2017).

In addition to *Pinus pinea* L., pine nuts are sourced from several other *Pinus* species worldwide, including Korean pine (*Pinus koraiensis*) from Northeast Asia, Chilgoza pine (*Pinus gerardiana*) native to Western Himalaya, Siberian pine (*Pinus sibirica*), Japanese pine (*Pinus pumila*), Chinese White pine *(Pinus armandii*), Mexican pine (*Pinus cembroides*), Bunge pine (*Pinus bungeana*), and Colorado pine (*Pinus edulis*) (Destaillats et al., 2010). Global production of consumable pine nuts averages around 30,000 tons, with the yearly average being 27,975 tons based on 2004 data, and 19,575 tons according to 2015 data (Yalim et al., 2022).

I.3.1. Pine nut composition

The chemical composition of pine nuts may vary slightly depending on the specific species of pine tree and their geographical region (Nergiz and Dönmez, 2004; Evaristo et al., 2010). Generally, pine nuts have a relatively high fat content (37.1-47.71 %), with oleic acid and linoleic acid being the main unsaturated fatty acids, and palmitic, stearic, and lignoceric acids as the primary saturated fatty acids. They are also a good source of proteins (31.6-35.5 %), providing essential amino acids essential for various bodily functions (Nergiz and Dönmez, 2004; Pereira et al., 2015; Lutz et al., 2017; Loewe-Muñoz et al., 2018; Zuleta et al., 2018), and they contain

carbohydrates (13.9 %) (Nergiz and Dönmez, 2004), including dietary fibers (11.66-14.6 %) (Nergiz and Dönmez, 2004; Nasri and Triki, 2007).

Moreover, pine nuts are rich in various vitamins, such as vitamin E, vitamin K, and B-complex vitamins like thiamine (B1) at 0.53 mg/100g, riboflavin (B2) at 0.19 mg/100g, niacin (B3), and folate (B9). Essential minerals found in pine nuts include magnesium (396 mg/100g), potassium (450 mg/100g), phosphorus (640 mg/100g), calcium (112 mg/100g), zinc (88.2 mg/100g), and sodium (50 mg/100g) (Nergiz and Dönmez, 2004; Kadri et al., 2015; Yalim et al., 2022).

I.3.2. Major protein composition

Examining the chemical composition of pine nuts reveals that protein constitutes the second major component, following fat. Proteins can be categorized into storage, structural, and biologically active proteins. The primary role of storage proteins is to provide essential proteins needed during seed germination (Chéreau et al., 2016). The classification of these proteins was accomplished using the Osborne and Campbell (1898) fractionation method, a biochemical technique that categorizes proteins based on their solubility in different solutions. This methosd distinguishes proteins into water-soluble proteins (albumins), proteins soluble in dilute salt solutions (globulins), proteins soluble in aqueous alcohol (glutelins), and proteins soluble in weakly acidic or alkaline solutions (prolamins).

Nasri and Triki (2007) carried out research focusing on the storage proteins of *Pinus pinea* L. nuts. The results of the study revealed that the storage proteins in pine nuts are predominantly composed of globulins, accounting for 75% of the total protein content. Further characterization of the globulin fraction using SDS-PAGE demonstrated the presence of several subunits with varying molecular weights (10 kDa, 12 kDa, 40 kDa, 50 kDa, and faint bands at approximately 75 kDa and 150 kDa). Globulins represent a diverse group of storage proteins that can be divided into two main types: 7S vicilin-type globulins and 11S legumin-type globulins. Both types of globulins are nutritionally significant due to their relatively low levels of cysteine and methionine (Chéreau et al., 2016).

Albumins were found to constitute approximately 15% of the total protein content in pine nuts' storage proteins, and they consist of three subunits with molecular weights of 14 kDa, 24 kDa, and 46 kDa. Glutelins, on the other hand, make up about 10 % of the total protein comprising four subunits, with the most abundant band at 43 kDa, along with others at 20 kDa, 8 kDa, and a

faint one at 64 kDa. Prolamins, in contrast, were present only in minimal amounts, accounting for approximately 1 to 2 % of the total protein content.

I.3.3. Amino acid composition

Plant proteins may lack certain essential amino acids, resulting in incomplete protein sources. Nuts, being rich in arginine, may be deficient in essential amino acids such as threonine, isoleucine, lysine, methionine, and cysteine. Notably, nuts have a high abundance of aspartic acid and glutamic acid, which are categorized as acidic amino acids. The low lysine/arginine ratio in nuts is attributed to their limited lysine content. Moreover, due to being protein-rich, nuts exhibit elevated levels of arginine and glycine (Yalim et al., 2022).

An analysis of the amino acid composition of Mexican pine nuts (*Pinus maximartinizii*) revealed that they contain 18 amino acids, notably encompassing all the essential ones (López-Mata, 2001) (table 3). Furthermore, Babich et al. (2017) conducted a similar analysis on the amino acid composition of Siberian pine nuts (*Pinus sibirica*), and the results are provided in Table III.

Table III: Amino acid profile of Siberian and Mexican pine nuts (López-Mata, 2001; Babich et al., 2017).

Amino acid	*Pinus sibirica*	*Pinus maximartinizii*		FAO/WHO
	g/100 g of protein	g/ 100 g of protein	g/16 g N	g/16 g N
Methionine*	1.66	1.27	1.95	2.5
Tryptophan*	1.18	0.37	0.57	1.1
Arginine*	15.41	10.96	16.73	-
Alanine	5.44	-	-	-
Lysine*	6.04	1.73	2.64	5.8
Leucine*	-	6.01	9.18	6.6
Isoleucine*	-	2.89	4.41	2.8
Valine*	3.37	3.02	4.60	3.5
Phenylalanine*	6.49	1.88	2.88	-
Histidine*	2.84	1.35	2.06	-
Cysteine	1.33	1.18	1.80	-
Threonine*	3.15	1.41	2.16	3.4
Tyrosine	2.86	2.76	4.22	6.3
Proline	5.47	1,82	2.78	-

Serine	6.72	3.71	5.66	-
Aspartic acid	5.89	6.83	10.43	-
Glutamic acid	11.84	7.05	10.75	
Glycine+ Alanine	-	6.07	9.26	-
Leucine + Isoleucine	15.73	-	-	-
Total aromatics	-	4.64	7.10	-
Total Sulphured	-	2.45	3.75	-

(*= essential amino acids)

I.3.4. Pine nut allergenicity

Allergies to tree nuts are widespread and have become a significant health concern due to increased availability. Tree nuts are among the eight most common allergens, and allergic reactions to them can be severe. Botanically, tree nuts are dry fruits consisting of an inedible hard shell and a seed. However, the term "tree nut" is commonly used to refer to any nut from a tree, even if it does not meet the botanical definition. Nine nuts, including walnut, almond, pistachio, cashew, pecan, hazelnut, macadamia, Brazil nut, and pine nut, account for the majority of tree nut allergies (Weinberger and Sicherer, 2018).

Allergy to tree nuts, particularly pine nut, has been described as particularly severe, and many cases involve patients sensitive to only one type of nut (monosensitized) (Cabanillas et al., 2016). Pine nut allergies were first documented in scientific literature in 1958, and severe anaphylactic reactions after consuming pine nuts make up the majority of reported cases (76 %). Monosensitivity to pine nuts is relatively high compared to tree nuts from other angiosperm species. Reactions have been reported after consuming pine nuts in various forms, including pesto sauce, salads, meatballs, cakes, candies, cookies, and pine nuts alone (Crespo et al., 2021).

Several research studies have focused on investigating the IgE-binding proteins present in pine nuts. The majority of allergens found in nuts are categorized as seed storage proteins, including vicilins (7S globulins), legumins (11S globulins), and 2S albumins. Additionally, certain nut allergens share similarities with proteins belonging to pathogenesis-related (PR) protein families, which are responsible for defending plants against pathogens (Mandal and Mandal, 2000). In the case of pine nuts, García-Menaya et al. (2000) identified a protein with a molecular weight ranging from 15 to 17 kDa. This protein was detected under non-reducing conditions and was found to trigger severe allergic symptoms in a patient after consuming pine nuts. Similarly,

Ibáñez et al. (2003) discovered a 17-kDa allergen, also detected under non-reducing conditions, which was responsible for inducing severe anaphylaxis in two patients who were monosensitived to pine nuts. The findings from these studies suggested that the recognition of these specific IgE-binding proteins was closely associated with the observed severe symptoms experienced by individuals after consuming pine nuts.

In other studies, researchers have reported the presence of another significant IgE-binding protein in pine nuts, with an approximate Mw of 50 kDa. This protein was also identified as a major allergen, and mass spectrometry analysis revealed it to be a vicilin. Vicilins have been acknowledged as major allergens in various nuts, including peanut, walnut, and pistachio. Moreover, other IgE-binding proteins of 30 kDa and 44 kDa were also detected in pine nut protein extracts through IgE-immunoblot (De las Marinas et al., 1998; Cabanillas et al., 2012; Novembre et al., 2012).

In a study conducted by Cabanillas et al. (2012), a 6-kDa protein was identified in pine nuts, detected under reducing conditions and which exhibited a molecular weight of 15-16 kDa under non-reducing conditions. This protein showed strong recognition by the serum of 55 % of patients with pine nut allergies, and notably, most of these patients had a history of severe anaphylaxis triggered by pine nuts. This suggests that these studies likely identified the same major allergen present in pine nuts. However, in a later study, Cabanillas et al. (2016) took a step further by purifying the primary allergen of pine nuts. Through g mass spectrometry analysis and database searches, they identified the allergen as a 2S albumin, officially named Pin p1 by the World Health Organization and International Union of Immunological Societies (WHO/IUIS). Pin p1 was identified as the major allergen in pine nuts from the *Pinus pinea* L. species. This allergen exhibited a compact structure with a conserved 3D motif linked by disulfide bonds, providing it with robust resistance to pH, enzymes, and heat. This resemblance to other allergenic 2S albumins found in more advanced species that produce tree nuts like hazelnuts, walnuts, cashews, or seeds like mustard seeds. Nevertheless, the amino acid sequence of Pin p1 displayed notable dissimilarity when compared to other allergenic 2S albumins. These substantial evolutionary distinctions between Pin p1 and other allergenic 2S albumins have been highlighted as a relevant factor contributing to the distinct characteristics of pine nut allergy.

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  1 MGVFSSPMST LRWVTLFAAL LSLLEWGTAH EDIVMDGDQV VQQQGRSCDP QRLSACRDYL
 61 QRRREQPSER CCEELQRMSP HCRCRAIERA LDQSQSYDSS TDSDSQDGAP LNQRRRRRGE
121 GRGREEEEAV ERAEELPNRC NLRESPRRCD IRRHSRYSII GGSD
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Figure 6: Amino acid sequence of the complete sequence of Pin p1 (Cabanillas et al., 2016).

Chapter II. Protein hydrolysates and bioactive peptides

Chapter II. Protein hydrolysates and bioactive peptides

Protein hydrolysates are defined as mixtures of polypeptides, oligopeptides, and free amino acids derived from protein sources through partial hydrolysis. The hydrolysis process can be achieved by enzymatic hydrolysis using food-grade proteolytic enzymes, chemical hydrolysis employing heat or suitable acids and alkalis, or microbial fermentation using proteolytic bacteria (McCarthy et al., 2013; Nasri, 2017).

Bioactive peptides are small fragments of food proteins consisting of 2-20 amino acids, with a molecular weight of less than 3 kDa. They are generated from the precursor protein from which they are encoded, and where their native structure is inactive (Korhonen and Pihlanto, 2003; Sarmadi and Ismail, 2010; Chalamaiah et al., 2019). These peptides are not active within the structure of the primary protein aka "parent protein" and require intact cleavage to exert their functions. Bioactive peptides are sometimes referred to as "cryptids", a term combining "cryptic" (hidden) and "peptide", particularly when they are encrypted within the parent proteins (Udenigwe, 2014).

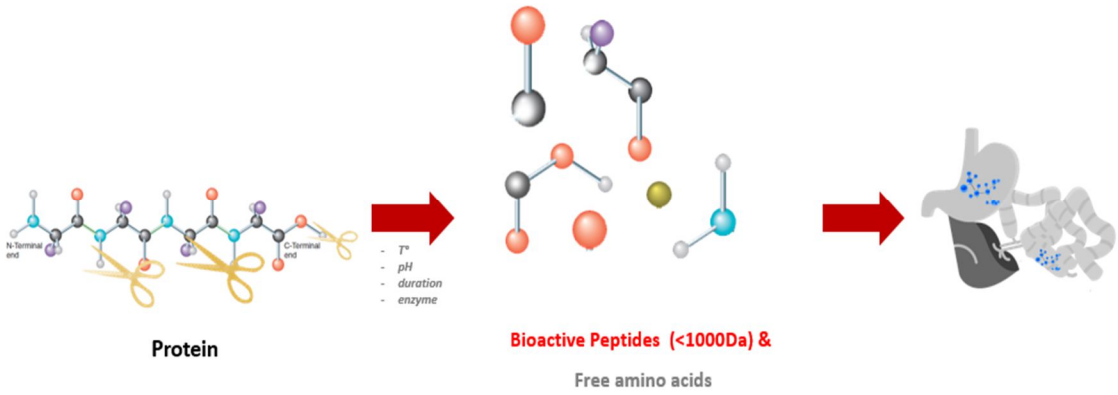

Figure 7: Enzymatic hydrolysis procedure to obtain bioactive peptides.

Bioactive peptides exert positive effects on bodily functions and contribute to human health, beyond their recognized nutritional value (Li-Chan, 2015). These peptides can regulate crucial bodily functions through a wide range of activities, including antihypertensive, antimicrobial, antithrombotic, immunomodulatory, opioid, antioxidant, and mineral binding functions. The specific biological activities of bioactive peptides depend on factors such as their structural properties, amino acid composition, charge, and sequence (Chalamaiah et al., 2012; Rizzello et al., 2016; Daliri et al., 2017; Chalamaiah et al., 2018).

Bioactive peptides have been produced from various food proteins. The primary sources of bioactive peptide precursors are milk and egg proteins, which constitute the largest category (Phelan et al., 2009; Power et al., 2013; Wada and Lönnerdal, 2014; Arrutia et al., 2016). However, proteins from meat, marine sources, and plants have also been used to obtain high-value-added ingredients (Lafarga and Hayes, 2014; Bah et al., 2016). Bioactive peptides derived from plant proteins serve a dual purpose by enhancing both health-related functions and the technological properties of foods (Görgüç et al., 2020).

In food formulation, bioactive peptides primarily consist of free amino acids and short-chain peptides (di- and tripeptides), and peptides with more than 12 amino acid subunits are generally not present (Clemente, 2000). Dipeptides and tripeptides are more bioavailable than free amino acids due to their lower hypertonicity, allowing easier absorption into cells with lower osmotic pressure differences. Some amino acids, like glutamine, tyrosine, and cysteine, cannot be directly absorbed in free form because of their low chemical stability or limited solubility in water (Kang et al., 2012).

II.1. Methods of production

Several approaches have been developed for the production of bioactive peptides, including chemical hydrolysis, enzymatic hydrolysis, and microbial fermentation (Korhonen and Pihlanto, 2006; Saadi et al., 2015). The optimal method possesses certain attributes such as affordability, industrialization potential, reproducibility, and compatibility with biological systems**.** Additional steps involving post-purification engineering or chemical production are necessary to synthesize peptides containing non-traditional amino acids or particular functional groups such as fatty acids or sugars. These peptides are generally used for medical purposes and are commonly employed as dietary supplements (Daliri et al., 2018; Akbarian et al., 2022).

II.1.1. Chemical hydrolysis

Chemical hydrolysis involves the breaking of peptide bonds using acid or alkali solutions. However, the use of strong acids or bases renders the chemical process environmentally unacceptable. Furthermore, the resulting products from chemical hydrolysis exhibit reduced nutritional qualities and biological activities due to the potential generation of undesirable by-products during non-specific chemical treatment. Acid hydrolysis, in particular, leads to the oxidation of cysteine and methionine, the destruction of certain serine and threonine residues, and the potential conversion of glutamine and asparagine to glutamate and aspartate, respectively

(Bucci and Unlu, 2000). Moreover, the bioactivities of protein hydrolysates obtained through chemical hydrolysis cannot be accurately reproduced since the cleavage of peptide bonds by chemical reagents lacks specificity. As a result, the hydrolysis process exhibits significant variations, leading to inconsistent bioactivity levels. Consequently, these drawbacks significantly limit the potential high-value applications of protein hydrolysates obtained by chemical hydrolysis (Nasri, 2017).

II.1.2. Microbial fermentation

Microbial fermentation is a biotechnological process used to obtain bioactive peptides by employing microorganisms capable of producing proteolytic enzymes that can hydrolyze proteins into shorter peptides (Onuh et al., 2014). The microorganisms commonly used are bacteria, fungi, or yeasts, which may be either naturally present in the substrate or added as a starter culture. Each type of microorganism has unique proteolytic systems that contribute to the production of bioactive peptides (Hernández-Ledesma et al., 2011). Lactic acid bacteria (LAB) are particularly noteworthy among bacteria and recognized among the most valuable microorganisms for obtaining bioactive peptides due to their high adaptability to various environments and substrates, their safety profile, since these bacteria are known as "friendly bacteria" because several strains are identified as "generally recognized as safe" (GRAS); and also, due to the efficient proteolytic system that characterizes them (Lafarga and Hayes, 2017; Melini et al., 2019). Some of the LABs mostly reported for their effective production of bioactive peptides are *Lactobacillus helveticus, Lactobacillus delbrueckii* ssp. *bulgaricus, Lactococcus lactis* ssp. *diacetylactis, Lactococcus lactis* ssp. *cremoris, and Streptococcus salivarius* ssp. *thermophylus* (Hernández-Ledesma et al., 2011).

To generate peptides with enhanced bioactivity, it is crucial to carefully handle the substrate, select suitable microorganisms, and maintain optimal environmental conditions such as pH, temperature, and humidity during the microbial fermentation process (Melini et al., 2019). The proteolytic system initiates when proteases associated with the cell envelope (CEP) break down proteins into oligopeptides. These oligopeptides, considered extracellular bioactive peptides, are not used for nitrogen assimilation by the proteolytic system. Active transporters then facilitate the movement of the oligopeptides into the cytoplasm. Finally, intracellular endopeptidases transform the oligopeptides into free amino acids or low molecular weight peptides, which contribute to the desired bioactive properties (Juillard et al., 2022).

II.1.3. Enzymatic hydrolysis

Enzymatic hydrolysis is the most common method for producing bioactive peptides from parent protein molecules (Oseguera-Toledo et al., 2014). It offers several advantages such as the absence of residual solvents or toxic chemicals in the final products, easy control of hydrolysis conditions, and the possibility of releasing a wide range of bioactive peptides. Key parameters such as pH, and temperature must be carefully monitored for optimal hydrolysis, as well as the hydrolysis duration, which is directly related to the degree of hydrolysis and has a direct impact on the size, amino acid composition, and bioactivities of the generated peptides (Kim and Wijesekara, 2010; Udenigwe and Aluko, 2012). Enzymatic hydrolysis can be performed using enzymes derived from microorganisms or plants, as well as digestive enzymes (Akbarian et al., 2022). To perform their functions, enzymes must bind to the substrate and catalyze the reaction. This is achieved by enzyme-specific active sites that form temporary bonds with the substrate, facilitating the cleavage process. The enzyme-substrate complex is stabilized by hydrogen bonds, hydrophobic bonds, or Van der Waals interactions (Cruz-Casas et al., 2021).

The most widely used enzymes for the production of bioactive peptides include pepsin, trypsin, and chymotrypsin, as well as commercial proteases such as Alcalase™, Protamex™, and Flavourzyme™ (Shahidi and Zhong, 2008). In addition, various combinations of proteases from different sources have also been employed to produce bioactive peptides from diverse proteins. Digestive enzymes are of particular interest due to their ability to mimic human digestion and assess the release of effective bioactive peptides (Sánchez and Vázquez, 2017; Zhang and Mu, 2017; Guan et al., 2018; Marciniak et al., 2018). The advantage of enzymatic digestion is that it yields protein hydrolysates whose amino acid composition is similar to that of the substrate protein, with slight modifications depending on the enzymes used (Nasri, 2017). Enzymatic hydrolysis can be carried out under traditional batch processing conditions, using immobilized enzymes, or ultrafiltration membranes, although the latter is less common due to disadvantages such as high cost and low yields (Cruz-Casas et al., 2021). Although enzymatic hydrolysis is regarded as a safe and promising method for the production of bioactive peptides, industrial implementation requires careful consideration of reaction time and enzyme quantities (Marciniak et al., 2018).

II.2. Biological activities of bioactive peptides

Over the past few decades, bioactive peptides have garnered considerable interest across various domains, being recognized for their potential as both nutritional supplements and

functional food, while also posing no significant harm to the human body. Presently, research has extensively explored the physiological effects of bioactive peptides, revealing their promising capacity to regulate a diverse array of bodily systems. The physiological impact of bioactive peptides is closely linked to their structural attributes, notably including the composition and sequence of amino acids, molecular weight, amino acid types at the N-terminal/C-terminal, hydrophobic/hydrophilic properties of the amino acid chain, and the charge property of the peptides' amino acids (Ye et al., 2022).

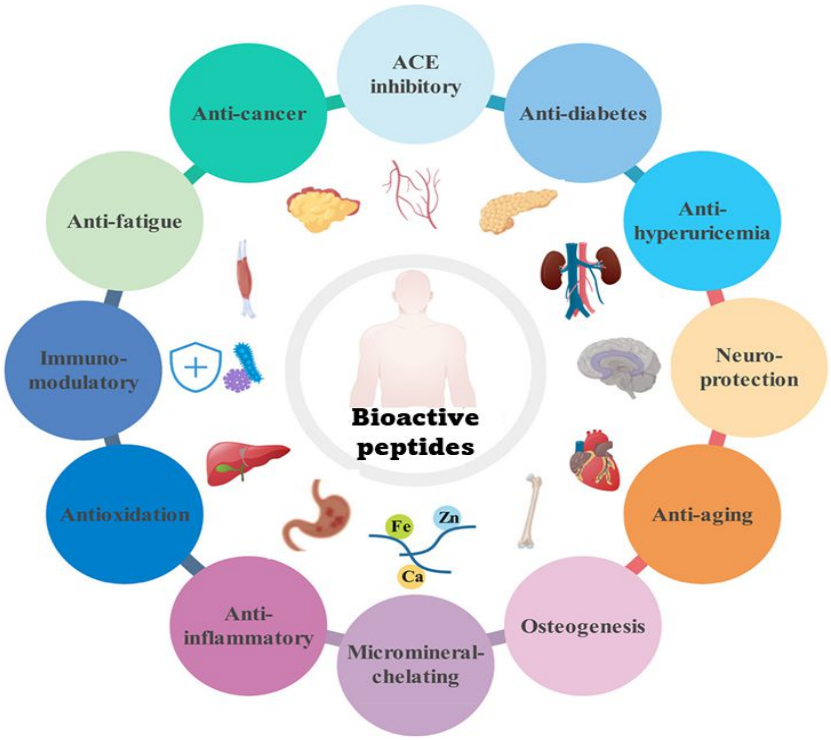

Figure 8: Different applications of bioactive peptides for humans.

II.2.1. Antioxidant activity

Free radicals, byproducts of normal aerobic respiration, react swiftly with other elements within the organism (Xie et al., 2013). These reactive oxygen species (ROSs) encompass superoxide anion, hydroxyl, lipoxyl, nitrogen dioxide, nitric oxide, and hydrogen peroxide (Jiang et al., 2020a), and are produced through various metabolic pathways, including cellular respiration, radiation, and light. Under typical physiological conditions, ROSs play crucial roles in immune responses and signal transmission. However, excessive ROS accumulation disrupts the body's natural balance, overwhelming the defense system's ability to neutralize them through enzymatic processes or non-enzymatic antioxidants (Xie et al., 2019). This leads to destructive actions,

damaging proteins, DNA, and membrane phospholipids, causing harm to normal cells and tissues, and contributing to diseases like heart disease, diabetes, dementia, Parkinson's, and cancer (Lobo et al., 2010). Synthetic antioxidants like BHA and BHT exist but are associated with toxic and carcinogenic effects (You et al., 2010b). Thus, research focuses on natural alternatives such as food protein-derived peptides, vitamin C, polyphenols, and polysaccharides to maintain redox homeostasis and balance oxidants and antioxidants (You et al., 2010b; Drach et al., 2011; Yu et al., 2018).

II.2.1.1. Mechanism of action

The mechanisms of action of antioxidant active peptides involve both physical and chemical processes. Physically, bioactive peptides can form a protective film or hinder direct contact with oxidants. Chemically, they eliminate free radicals, act as proton donors, or function as metal ion chelators to exert their antioxidant capacity (Ye et al., 2022).

The overall antioxidant effects of protein hydrolysates are likely the result of several collaborative mechanisms, encompassing metal ion chelation, scavenging of free radicals, inhibition of lipid peroxidation, oxidase inhibition, antioxidant enzyme cofactors, and singlet electron transfer (such as reducing capacity) (Sampath Kumar et al., 2012).

The antioxidant capacity of bioactive peptides depends on various factors, such as their amino acid composition, sequence, and molecular weight. Specific amino acids like cysteine, lysine, histidine, methionine, tryptophan, and tyrosine demonstrate effective scavenging of free radicals (Udenigwe and Aluko, 2012) . Peptides containing cysteine, histidine, aspartic acid, and glutamic acid enhance metal chelation ability, crucial for inhibiting free radical formation (Guo et al., 2014). Aromatic amino acids such as Trp, Tyr, His, and Phe possess phenol, indole, and imidazole groups, respectively, enabling them to donate hydrogen radicals to electron-deficient free radicals (Sarmadi and Ismail, 2010; Duan et al., 2014). Histidine, with its imidazole ring, exhibits significant potential for scavenging free radicals (Saidi et al., 2014). Moreover, the antioxidant characteristics of peptides were linked to the existence of hydrophilic amino acids like proline, alanine, valine, and leucine at the N-terminal position, as well as amino acids such as tyrosine, valine, methionine, leucine, isoleucine, glutamine, and tryptophan at the C-terminal position (Ye et al., 2022).

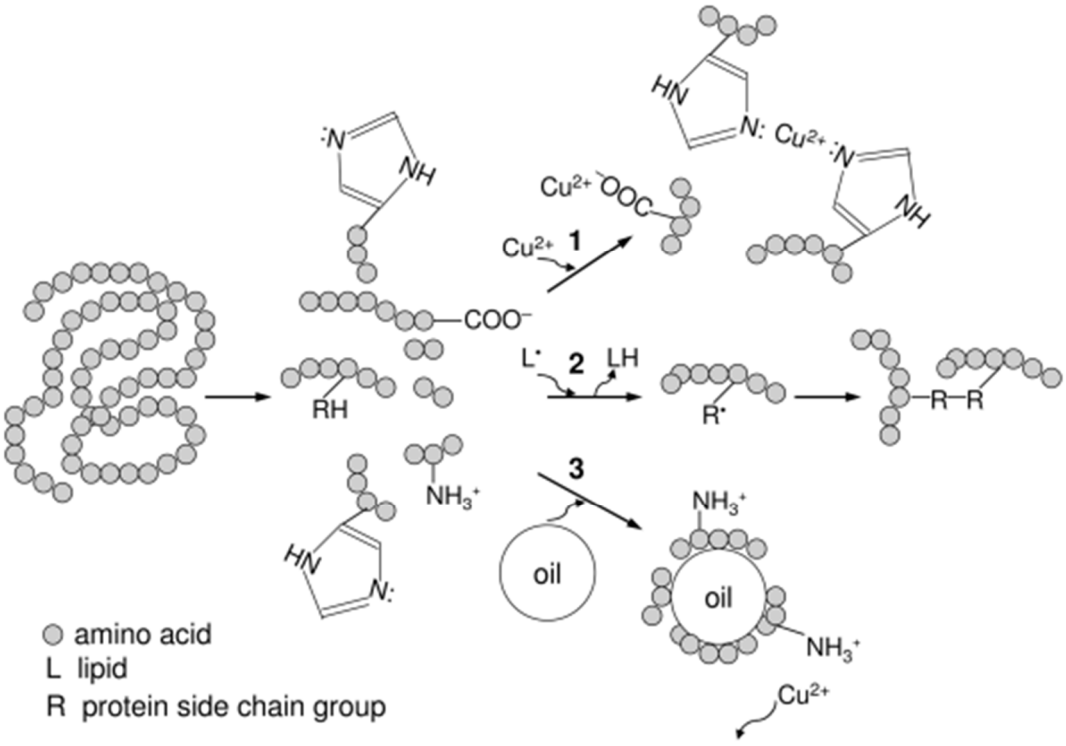

Figure 9: Schematic representation of chemical and physical mechanisms of antioxidant peptides to inhibit oxidative processes. (1) Metal chelation; (2) radical scavenging; (3) physical hindrance (shielding; repulsion) (Xiong, 2010).

Most antioxidant peptides obtained from food contain hydrophobic amino acids like valine or leucine at the N-terminal of their sequence, along with proline, histidine, tyrosine, tryptophan, methionine, and cysteine in their structure. The inclusion of hydrophobic amino acids, such as valine or leucine, enhances the peptides' ability to interact with the fat phase, thereby enabling easier access to free radicals generated in the fat phase (Ranathunga et al., 2006; Zou et al., 2016).

Furthermore, the antioxidant activity of peptides is influenced by their molecular weight. In general, most antioxidant peptides consist of 4 to 16 amino acid units and have a molecular weight ranging from 0.4 to 2 kDa. The molecular weight of peptides influences the pathway to target sites and modulates the gastrointestinal digestion mechanism, which in turn may enhance antioxidant capacity *in vivo* (Toldrá et al., 2018). As research demonstrated, focusing on corn gluten protein hydrolysates, peptides within the molecular weight range of 500-1500 Daltons exhibit greater antioxidant potency compared to those with higher or lower molecular weights. The enhanced efficacy of smaller peptides is attributed to their easier interaction with free radicals and more efficient radical elimination. However, it is noteworthy that a higher degree of hydrolysis

leads to reduced antioxidant activity due to the breakdown of peptides into amino acids with limited or negligible antioxidant capabilities (Ranathunga et al., 2006; Li et al., 2008; Aluko, 2015).

Table IV: Antioxidant activities of some nut-derived peptides.

Protein source	Enzymes used to produce the peptides	Tested antioxidant activity	Peptide sequence	References
Walnut meal protein	Pepsin, pancreatin	ABTS radical scavenging activity, and ORAC assay	Thr-Tyr; Ser-Gly-Gly-Tyr	(Feng et al., 2019)
Walnut protein isolate	Neutrase, Alcalse, pepsin	DPPH and hydrolxyl radical scavenging activity, FRAP, Fe^{2+} chelating activity, linolic acid peroxidation inhibition	Ala-Asp-Ala-Phe	(Chen et al., 2012)
Defatted peanut meal	Protease	DPPH radical scavenging activity, FRAP, ORAC assay, Fe^{2+} chelating activity	Tyr-Gly-Ser	(Zheng et al., 2012)
Chinese chestnut protein isolate	Alcalase	DPPH, ABTS, and hydroxyl radical scavenging activity	Val-Tyr-Thr-Glu Thr-Lys-Gly-Gln Met-Met-Leu-Gln-Lys Thr-Pro-Ala-Ile-Ser Val-Ser-Ala-Phe-Leu-Ala	(Feng et al., 2018)
Pecan meal	Alcalase	DPPH, ABTS and Hydroxyl radical scavenging activity, Fe^{2+} chelating activity, FRAP	Leu-Ala-Tyr-Leu-Gln-Tur-Thr-Asp-Phe-Glu-Thr-Pro	(Hu et al., 2018)
Korean pine nut peptide	/	DPPH radical scavenging activity, ORAC assay	Glu-Asp-His-Cys-His	(Zhang et al., 2019)
Korean pine nut peptides	/	DPPH radical scavenging activity and cellular antioxidant activity	Gln-Cys-His-Lys-Pro Gln-Cys-His-Gln-Pro Lys-Cys-His-Gln-Pro Lys- Cys-His-Lys-Pro	(Lin et al., 2017)
Korean pine nut meal	Alcalase	DPPH and ABTS radical scavenging activity, FRAP, cellular antioxidant activity assay	Lys-Trp-Phe-Cys-Thr Gln-Trp-Phe-Cys-Thr	(Yang et al., 2017)

II.2.2. Immunomodulating activity

An immunomodulator refers to any substance capable of enhancing, reducing, or modifying immune responses by altering any component of the immune system, encompassing both innate and adaptive functionalities of the immune system. The immune system is crucial for our survival as it defends against pathogens, but it can be influenced by factors like stress, unhealthy habits, pathogens, and antigens (Segerstrom and Miller, 2004). Some drugs like cyclosporine, tacrolimus, glucocorticoids, phytol, aristolochic acid, plumbagin, and levamisole have been successfully used to modulate the human immune response (Gertsch et al., 2011). Nevertheless, the high cost and toxic side effects of allopathic drugs limit their usage in patients, making them less suitable for chronic or preventive purposes (Wang et al., 2010).

In recent research, it has been found that dietary components can effectively modulate immune function, and the discovery of novel immune-modulating peptides derived from food proteins offers promising benefits for dietary treatments. Parker et al. (1984) made the initial discovery of an immunomodulatory peptide in human casein enzymatic hydrolysate. Since then, various studies have reported the immunomodulatory activities of different food protein hydrolysates or peptides. These food-derived peptides have demonstrated diverse effects on both innate and adaptive immune responses. They can stimulate the production of cytokines and antibodies, promote lymphocyte proliferation, enhance macrophages' ability to engulf pathogens, increase natural killer cell activity, strengthen the body's defense against invading pathogens, and suppress pro-inflammatory responses of host cells to bacterial components like lipopolysaccharide (LPS) (Duarte et al., 2006; Hou et al., 2012; Cai et al., 2013; Chalamaiah et al., 2015; Wu et al., 2016; Toopcham et al., 2017). The mechanism behind these effects is believed to involve the direct binding of food-derived peptides to receptors on the surface of immune cells. Importantly, while these immunomodulatory peptides do not directly interact with pathogens, they significantly contribute to the host's defense response (Maestri et al., 2016).

Recently, researchers have identified immunomodulatory peptides from various hydrolyzed food proteins**.** The immunomodulatory potential of these peptides is influenced by their amino acid composition, sequence, length, charge, hydrophobicity, and molecular structure (Berthou et al., 1987; Jacquot et al., 2010)). These food-derived peptides with immunomodulatory effects are generally short (2–10 residues) and hydrophobic. They often contain hydrophobic amino acids such as Gly, Val, Leu, Pro, Phe, as well as negatively charged amino acids like Glu

and aromatic amino acids like Tyr. Studies have indicated that hydrophobic amino acids, along with glutamine, glutamic acid, tyrosine, tryptophan, cysteine, asparagine, and aspartic acid residues, contribute to the immunomodulatory activities of these peptides (Hou et al., 2012; Lee et al., 2012; Kim et al., 2013; Vo et al., 2013; He et al., 2015).

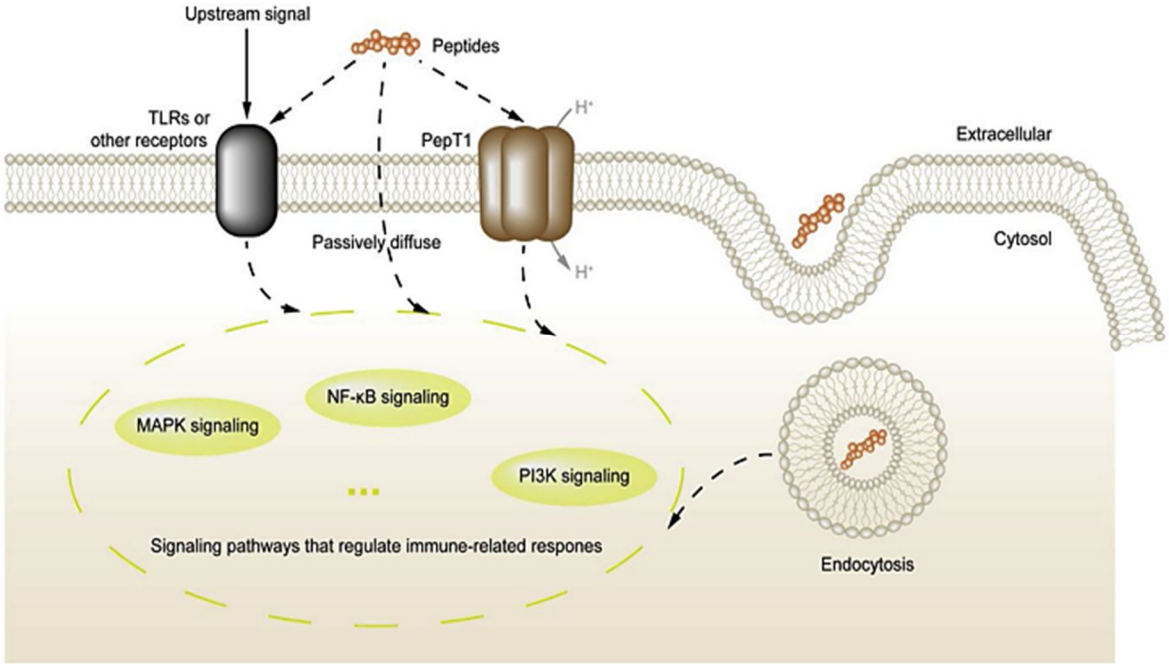

Figure 10: mechanisms of immunomodulatory peptides. The immunomodulatory effects of peptides can occur through various mechanisms. These peptides can directly affect receptors (e.g. TLRs), or enter cells via passive diffusion, peptide transporter (PepT1, located in intestinal epithelial cells, responsible for transporting small peptides such as di- and tri-peptides), and fluid-phase endocytosis. Following these processes, they can disrupt inflammatory signaling pathways such as NF-kB, MAPK and PL3K (Cai et al., 2020a).

These immunomodulatory peptides have diverse targets, including monocytes, macrophages, natural killer (NK) cells, mast cells, T and B lymphocytes, CD4+ and CD8+ T cells, as well as CD49b+, CD11b+, and CD56+ cells. Although the exact mechanisms of their immunomodulatory effects are not fully understood, evidence suggests that they mainly function through the activation of macrophages, stimulation of phagocytosis, increased leukocyte count, induction of immune modulators like cytokines, NO, and immunoglobulins, stimulation of NK cells, splenocytes, CD4+, CD8+, CD11b+, and CD56+ cells, activation of transcription factors

like nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, as well as the inhibition of pro-inflammatory mediators (Cian et al., 2012; Chalamaiah et al., 2014; Ahn et al., 2015). Thus, depending on their amino acid sequence, composition, length, and structure, these immunomodulatory peptides can regulate both innate and adaptive immune responses by binding to specific receptors on the surface of target cells (Masotti et al., 2011; Rodríguez-Carrio et al., 2014).

Research on food-derived peptides or protein hydrolysates has extended to exploring their inflammation-related immunomodulatory potential, given the significant role of inflammation in the pathophysiology of various diseases like cancer, atherosclerosis, rheumatoid arthritis, ulcerative colitis, asthma, and diabetes. Studies have shown that several peptides or protein hydrolysates from food proteins could modulate inflammation by inhibiting the production of inflammatory mediators such as nitric oxide (NO), prostaglandin E2 (PGE2), and cytokines (IL-1β, IL-6, IL-8, and TNF-α) in *in vitro* cell culture models stimulated with bacterial lipopolysaccharide (LPS). Macrophages, which are activated by Toll-like receptor ligands like LPS, are commonly used in *in vitro* models to investigate the anti-inflammatory potential of food-derived peptides. These activated macrophages release various inflammatory mediators, and the food-derived peptides have been shown to inhibit these inflammatory responses through different molecular mechanisms, including inhibition of cytokines, cyclooxygenases, inducible nitric oxide synthase (iNOS), MAPKs, and the transcription factor NF-κB (Ahn et al., 2015; Karnjanapratum et al., 2016; Sae-leaw et al., 2016).

II.2.3. Antihypertensive activity

High blood pressure is on the rise in developed countries and poses a significant risk for serious health conditions such as myocardial infarction, congestive heart failure, stroke, arteriosclerosis, and end-stage renal disease (Kannel and Higgins, 1990). The key regulator of blood pressure is the Angiotensin-I converting enzyme (ACE), responsible for converting angiotensin-I into angiotensin-II, a potent vasoconstrictor. Blocking ACE activity is a crucial approach in preventing hypertension (Shahidi and Zhong, 2008). Various natural ACE inhibitory peptides have been identified in different food proteins, including cod frame, pollack skin, sea bream scales, yellow-tail bone and scales, yellow sole frame, tuna frame, clam, krill, mussel, oyster, and shrimp (Hartmann and Meisel, 2007). This discovery has led to a growing interest in using bioactive peptides for the prevention and initial treatment of mild hypertension (Guang and

Phillips, 2009).

Different anti-hypertensive peptides have been evaluated for their ACE activity competitiveness through kinetic analysis using Lineweaver–Burk plots (Zhao et al., 2009). The mechanism of action of these peptides differs from synthetic drugs, as they interact with ACE in a competitive manner rather than indiscriminately blocking its action. While synthetic drugs directly inhibit ACE, anti-hypertensive peptides compete with it during the conversion of angiotensin-I to angiotensin-II by ACE (Ahhmed and Muguruma, 2010).

Anti-hypertensive peptides function by relaxing arterial walls and reducing fluid volume, leading to the inhibition of angiotensin-II formation. Consequently, these peptides improve heart function and enhance blood and oxygen flow to vital organs such as the heart, liver, and kidneys (Ahhmed and Muguruma, 2010). Studies have shown that peptides containing tryptophan, tyrosine, phenylalanine, or proline at the C-terminal, and branched-chain aliphatic amino acids at the N-terminal, are effective in binding to ACE as competitive inhibitors (Li et al., 2004).

Additionally, certain peptides have been observed to follow a non-competitive mechanism (Suetsuna and Nakano, 2000), wherein they form a dead-end complex with the enzyme, irrespective of whether a substrate molecule is bound or not. The hydrophobicity of the N-terminus, a common characteristic of ACE inhibitory peptides, likely contributes to their inhibitory activity (Rho et al., 2009). These ACE inhibitory peptides are typically short-chain peptides, often containing polar amino acid residues like proline. Moreover, the structure-activity relationships among various peptide ACE inhibitors suggest that the C-terminal tripeptide sequence of the substrate greatly influences the binding to ACE, with peptides containing hydrophobic amino acids at these positions displaying potent inhibitory effects (Qian et al., 2007).

II.2.4. Antimicrobial activity

In the last two decades, a multitude of antimicrobial peptides with antibacterial, antiviral, and antifungal have been discovered in both vertebrates and invertebrates, playing a crucial role in the innate immune system of hosts. These peptides have distinct mechanisms of action compared to traditional antibiotics, making them promising candidates for fighting infections (Sato et al., 2006).

Antimicrobial peptides typically have common characteristics, consisting of less than 50 amino acids, with about half being hydrophobic (Rydlo et al., 2006). Those containing essential amino acids like lysine and arginine display the highest antimicrobial effectiveness. Cationic and

amphipathic amino acids also contribute to their antimicrobial activity (Jin et al., 2005; Chou et al., 2008). Some of these peptides, known as cell-penetrating peptides, possess the ability to transport diverse materials through cell membranes, including drugs, proteins, liposomes, and nanoparticles (Layek et al., 2015). Certain antimicrobial peptides indirectly combat harmful microbes by interacting with the host immune system (Wittkopf et al., 2014). For instance, milk protein hydrolysate stimulates the host's immune response, enhancing natural killer cell proliferation, macrophage phagocytosis, and the expression of antibodies, cytokines, and chemokines (Kayser and Meisel, 1996).

II.2.5. Blood-lipid lowering activity

Hyperlipidemia, especially elevated cholesterol, poses a significant risk for cardiovascular disease. Numerous research studies have shown that peptides derived from soy can be highly effective in reducing blood cholesterol levels, both in animal experiments and human trials. Soy-rich diets have emerged as a powerful dietary approach for managing high cholesterol (Ros, 2000). Some specific soy-derived peptides, like LPYPR and IAVPGEVA, share structural similarities with endostatin and VPDPR, and have demonstrated their ability to lower cholesterol levels. These peptides achieve this by inhibiting the activity of 3-hydroxy-3-methylglutaryl-coenzyme reductase, a crucial enzyme involved in cholesterol synthesis (Möller et al., 2008). Additionally, hydrophobic peptides derived from soy proteins can interact with bile acids, promoting the excretion of fatty acids through the feces (Korhonen and Pihlanto, 2006; Möller et al., 2008).

Milk is another valuable source of bioactive peptides known for their cholesterol-lowering effects. Researchers have identified a cholesterol-reducing peptide in digested beta-lactoglobulin hydrolysate found in milk (Nagaoka et al., 2001).

Interestingly, the effectiveness of these peptides in reducing hyperlipidemia appears to be influenced by their amino acid composition. Peptides with lower proportions of methionine, glycine, lysine, and arginine have shown greater efficacy in combating high cholesterol. In contrast, bovine casein protein, which contains higher levels of these specific amino acids, particularly methionine and glycine, has been associated with increased cholesterol levels (Nagaoka et al., 2001).

II.2.6. Anti-obesity activity

Peptides have the ability to influence nutrient absorption, particularly in the small intestine, leading to a decrease in appetite. Numerous research studies have demonstrated that peptides

obtained from dietary proteins can transmit signals of satiety to the brain, effectively curbing the desire for further food consumption (Nagaoka et al., 2001). Notably, peptides derived from casein have been found to regulate eating behavior by activating the cholecystokinin A (CCK-A) receptor in the body (Pupovac and Anderson, 2002).

II.2.7. Anti-diabetic activity

Various peptides derived from plants offer potential benefits for individuals with diabetes, operating through diverse pathways. Some of these pathways that have been investigated include the ability to inhibit alpha-amylase, dipeptidyl peptidase IV, the glucose transporter system, and even mimic insulin activity (Patil et al., 2020).

II.2.8. Anti-aging activity

As we age, the production of extracellular matrix proteins decreases, while their breakdown increases, leading to reduced skin elasticity and the appearance of aging signs like wrinkles and fine lines. Matrikines, which are small peptides resulting from the breakdown of these proteins, play a role in regulating cellular functions such as proliferation and differentiation (Sivaraman and Shanthi, 2018; Leroux et al., 2020).

Peptides offer diverse skin-rejuvenating benefits, including stimulating collagen production, promoting wound healing, reducing wrinkles, and providing antioxidant and antibacterial effects. They are extensively used in cosmetics to enhance skin health (Fields et al., 2009; Rahnamaeian and Vilcinskas, 2015). Cosmetic peptides function through various pathways, modulating collagen levels, decreasing IL-6 secretion, and stimulating collagen and elastin synthesis. For instance, peptides like Val-Gly-Val-Ala-Pro-Gly stimulate skin fibroblasts and boost skin angiogenesis, while Nonapeptide-1 inhibits tyrosine enzyme activity (Katayama et al., 1991; Field et al., 2018; Maeda et al., 2018).

Certain peptides act as inhibitors of neurotransmitters, resembling the mechanism of Botox® or Dysport®, reducing muscle contractions and wrinkles by hindering neurotransmitter signals. However, the challenge lies in effectively delivering these peptides from the outer skin layers to the deeper living parts (Kluczyk et al., 2021).

II.3. Bioavailability of bioactive peptides

To exert physiological bioactivities, peptides must maintain their structural integrity during digestion and transportation. However, compared to intentionally designed drugs, peptides have considerably lower oral bioavailability (Adessi and Soto, 2002). When orally consumed, peptides

interact with the gastrointestinal tract, facing stomach and intestinal proteases and peptidases that can hydrolyze the peptides, resulting in the loss or alteration of their bioactivity. In peptide drug design, this issue is addressed through chemical modifications that safeguard vulnerable peptide bonds from being cleaved by endogenous peptidases (Adessi and Soto, 2002; Udenigwe and Fogliano, 2017).

While ample evidence confirms the *in vitro* antioxidant activity of bioactive peptides, establishing a direct relationship between their *in vitro* antioxidant properties and their *in vivo* antioxidant capacity presents challenges. Peptides undergo degradation and modification in the intestine, vascular system, and liver, which hinders the straightforward consideration of their *in vivo* antioxidant effects. Overcoming these barriers and reaching their target in an active form is crucial for peptides (Sarmadi and Ismail, 2010).

Although only a small portion of bioactive peptides manage to pass the intestinal barrier, they can still induce biological effects at the tissue level, even if their nutritional significance is usually limited. The intact absorption of peptides represents a normal physiological process that differs from the regular peptide transporter route (Gardner, 1988). Several mechanisms, including the paracellular route, passive diffusion, transport via carriers, endocytosis, and the lymphatic system, contribute to the intact absorption of peptides. Peptides and proteins have the ability to escape digestion and be absorbed in their intact form through the interstitial space into the intestinal lymphatic system. However, the permeability of compounds through the capillary of the portal circulation and their lipid solubility affects their entry into the intestinal lymphatic system (Wasan, 2002; Sarmadi and Ismail, 2010).

The absorption route of bioactive peptides depends on the number of amino acid residues they contain. Peptides with 2 or 3 amino acid residues can enter enterocytes and renal epithelia through PepT1 and PepT2 transporters. PepT1 is a low-affinity transporter for short-chain peptides with neutral charge and high hydrophobicity, while PepT2 is a high-affinity transporter. For peptides with more than three or four residues, nonreceptor-mediated or receptor-mediated endocytosis, such as transcytosis or paracellular diffusion, is utilized. The transport mechanism is also influenced by the amino acid composition of the peptides, with large hydrophobic peptides (four to nine amino acids) being able to flow passively through cell membranes (Patil et al., 2022).

Molecular size and structural properties, such as hydrophobicity, influence the primary transport route for peptides (Shimizu et al., 1997). Research findings suggest that peptides

containing 2-6 amino acids are more easily absorbed compared to proteins and free amino acids (Grimble, 1994). Both small peptides (di- and tripeptides) and large peptides (10-51 amino acids) can cross the intestinal barrier intact and exhibit their biological functions at the tissue leve. However, as the molecular weight of peptides increases, their chances of successfully passing the intestinal barrier decrease (Roberts et al., 1999). Peptides containing proline and hydroxyproline demonstrate resistance to digestive enzymes, particularly tripeptides with Pro-Pro at the C-terminal, which are impervious to proline-specific peptidases (FitzGerald and Meisel, 2000). A study revealed that the amount of peptide in human plasma increases in a dose-dependent manner, suggesting that saturation of peptide transporters could influence the quantity of peptides entering the peripheral blood (Matsui et al., 2002).

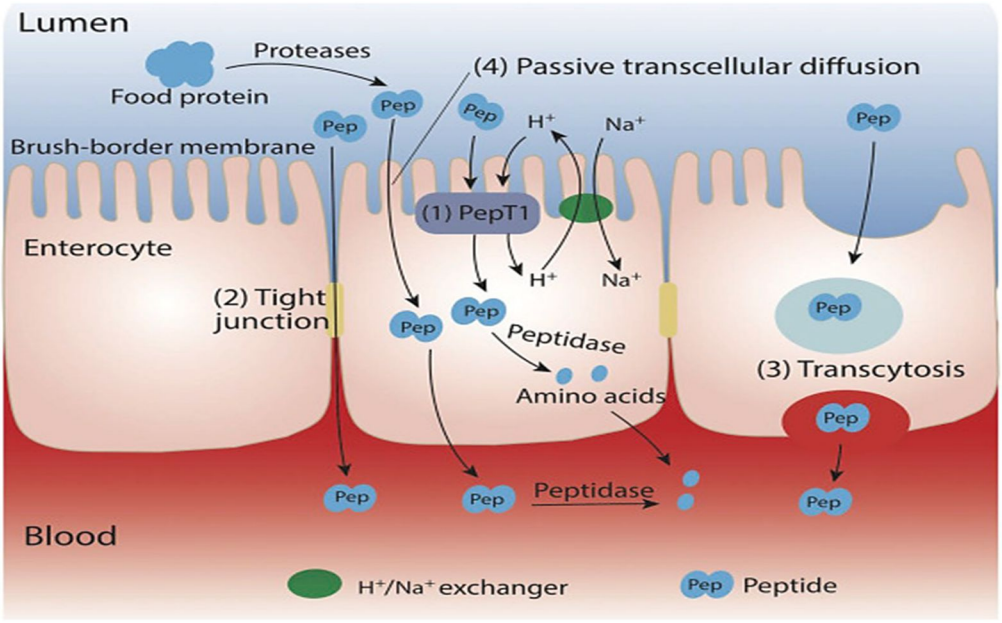

Figure 11: Bioavailability of food-derived bioactive peptides across the intestinal epithelial membrane (Lu et al., 2021).

Due to the incomplete bioavailability of peptides after oral ingestion, a peptide with potent antioxidant activity *in vitro* may exhibit minimal or no activity *in vivo*. However, other bypass routes that enhance peptide absorption can mitigate this limitation. Conversely, *in vivo* antioxidant activity might exceed *in vitro* activity. In such cases, bioactive peptides may exhibit their biological functions through mechanisms different from those observed in experiments. Additionally, the robust *in vivo* activity could be attributed to increased peptide activity following their breakdown by gastrointestinal proteases (Li et al., 2004; Erdmann et al., 2008).

II.4. Safety of bioactive peptides

Bioactive peptides offer various health benefits, but it is important to carefully assess their potential adverse effects before incorporating them into consumable products. There have been limited studies on the harmful effects of food-derived bioactive peptides on human health. Some peptides have demonstrated cytotoxic effects, which make them potential candidates for anticancer agents (Hartmann et al., 2007). Additionally, concerns exist regarding the allergenic properties of certain peptides, as proteins present in foods and pollens are often sources of allergenic substances. Although hydrolysis of proteins into bioactive peptides reduces their allergenic properties, some peptides may still retain the allergenic potential from their parent proteins (Hartmann et al., 2007).

The production of bioactive peptides by fermentation and enzymatic hydrolysis can lead to the formation of toxic peptides. For example, hypoallergenic formulas produced using enzymatic hydrolysis may contain toxic compounds that can still trigger allergic reactions in newborns. Even after hydrolysis, certain peptides in milk formulas retain their allergenic activity (Sampson et al., 1991).

Peptides derived from dietary proteins may possess toxic properties, as evidenced by naturally occurring toxic peptides found in mushrooms (Khan et al., 2018). In addition, certain protein families, such as lectins and ribosome-inactivating proteins, exhibit antitumor and antiviral abilities, but they also have potential toxicity (Dang and Van Damme, 2015).

Although bioactive peptides from food sources have been safely consumed for long periods, further research is essential to ensure their safety, considering the possibility of cytotoxic effects (Sarmadi and Ismail, 2010).

Part II. Research problems, hypotheses, and objectives

I. Research problems

The food industry is actively pursuing plant-based alternatives to animal-derived ingredients due to various reasons such as limited animal protein availability, consumer concerns, religious constraints, dietary choices, and meal preferences involving animal-based components. These substitutes are expected to closely mimic the nutritional and functional qualities of animal proteins (Karaca et al., 2011a; Mohan and Mellem, 2020a). Capitalizing on this trend, protein-rich ingredients of plant origin present a valuable source for crafting exceptionally versatile components. One such example is pine nuts, renowned for their substantial protein content (31.6-35.5%) and valuable nutritional and organoleptic attributes. The consumption of pine nuts offers numerous health benefits, including reduced cardiovascular risks, reduced risk of type 2 diabetes, and reduced body mass index (Nergiz and Dönmez, 2004; Evaristo et al., 2010; Loewe-Muñoz et al., 2018)

Methodologies employed to extract proteins have a perceptible impact on both protein composition and functionality. Commonly used in the food industry, alkaline extraction and isoelectric precipitation parameters, namely pH, extraction time, and solvent-to-sample ratio, can distinctly shape the structural and functional attributes of the resulting isolates. The pH parameter, in particular, plays an essential role, as it has the ability to induce conformational changes in the protein structure, consequently imparting modifications to its functional properties (Gao et al., 2018; Cai et al., 2020b; Cui et al., 2020).

Interest in food-derived bioactive peptides has grown due to their safer and gentler effects in the prevention or management of human diseases (Akbarian et al., 2022). These peptides have considerable potential as functional foods, natural health products, and nutraceuticals (Zaky et al., 2022). Among these, antioxidant peptides have received increasing attention due to their significant contribution to the prevention and treatment of oxidative stress-related diseases The efficacy of these bioactive peptides can be influenced by various factors, such as protein source, protease type, degree of hydrolysis, amino acid composition, and peptide structure (Memarpoor-Yazdi et al., 2013). Consequently, the judicious selection of appropriate proteases to initiate enzymatic hydrolysis is of paramount importance.

The primary concern regarding consuming pine nuts and their related products revolves around their potential to induce allergies. A key player in this allergenic response is Pin p1, a

protein band with a molecular weight of 6 kDa, identified as the major allergen in pine nuts (Cabanillas et al., 2016). The allergenic nature of food allergens, in general, is closely tied to specific antigenic epitopes, including linear and conformational forms (Xi et al., 2021). Current insights into reducing allergenicity involve disrupting activity or causing loss, with newly exposed epitopes being fewer than disrupted or inactive ones. Diverse processing techniques, including enzymatic hydrolysis, can modify allergens to various extents (Shriver and Yang, 2011). Enzymatic hydrolysis not only alters conformational antigen epitopes through proteolysis but also cleaves specific amino acid residues to eliminate linear antigen epitopes, thereby reducing protein sensitization (Toomer et al., 2015; Oliveira et al., 2019).

II. Research hypotheses

II.1. Hypothesis 1

Employing surface response methodology will enable the selection of optimal extraction parameters to produce protein isolates with high purity and improved functional properties.

II.2. Hypothesis 2

The use of various proteases, either individually or sequentially, enables the generation of diverse protein hydrolysates, each with distinct characteristics in terms of sequence, bioactivity, and mechanism of action.

II.3. Hypothesis 3

When subjected to enzymatic hydrolysis, the allergenicity in protein hydrolysates will reduce and might exert a hypoallergenic effect. Furthermore, simulated *in vitro* gastrointestinal digestion may affect the antioxidant activity of protein hydrolysates.

III. Research Objectives

To address the research hypotheses, the study will be carried out according to four major objectives:

Objective 1: Optimize extraction conditions (pH, extraction time, sample-to-solvent ratio) using RSM to obtain a protein isolate with high purity and improved functional properties (solubility and emulsifying activity). Then, evaluate the functional properties and perform biochemical analyses on the protein isolate obtained under optimal extraction conditions.

Objective 2: Apply enzymatic hydrolysis using different proteases on the optimal protein isolate and evaluate the antioxidant activity of the generated protein hydrolysates *in vitro* using

different assays. Characterize the protein hydrolysates with the most potent bioactivity using tandem mass spectrometry (MS/MS).

Objective 3: Investigate the bioavailability of the one selected protein hydrolysate (trypsin hydrolysate) and protein isolate and using simulated gastrointestinal digestion.

Objective 4: Examine the allergenicity of the protein isolate and trypsin hydrolysates using specific immunological techniques (IgE-Elisa inhibition and western blot analysis).

Part III. Experimental study- Results and discussion

Chapter I. Optimization of alkali extraction conditions of Pinus pinea L. nuts protein isolates, evaluation of their functional properties and biochemical characterization

Chapter I. Optimization of alkali extraction conditions of *Pinus pinea* L. nuts protein isolates, evaluation of their functional properties and biochemical characterization

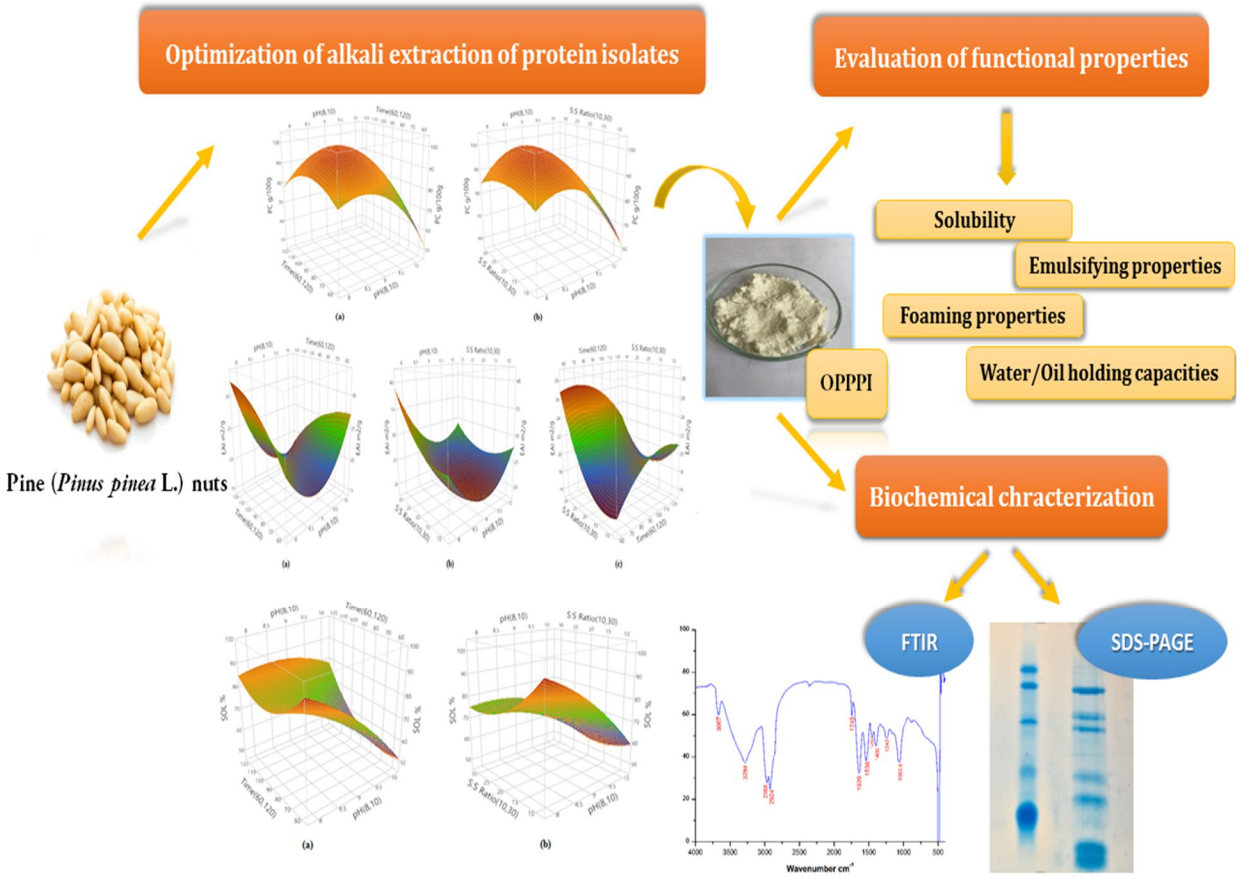

Figure 12: Graphical abstract representing the optimization of alkali extraction, evaluation of functional properties, and biochemical characterization of pine nuts protein isolates.

Abstract

Pinus pinea L. nuts are a valuable source of protein and can be regarded as a potential source of plant-based proteins applied in the food industry. The main objective of the present study was to investigate the influence of alkali extraction conditions, namely extraction pH, extraction time and solvent-to-sample ratio on the purity of the obtained protein isolates and their functionalities (solubility and emulsifying activity). This investigation was conducted using response surface methodology (RSM). Subsequent analyses were carried out to assess the functionality of the optimized protein isolates (emulsion stability, water/oil holding capacities and foaming capacity and stability) as well as their composition and secondary structure. The results show that maximum purity (89.13 g/100 g of extract), solubility (91.16%) and emulsifying activity index (EAI) (28 m^2/g) were obtained under the optimal conditions, which were determined to be extraction pH 8, extraction time of 108 min, and solvent-to-sample ratio 1:10 g/mL. FTIR spectroscopy analysis showed that the β-sheet was the main secondary structure in the pine nuts protein isolate. In addition, SDS-PAGE analysis revealed that the protein isolate was composed mainly of 11S globulin, 7S globulin and 2S albumin. Results indicated that OPPPI had appreciable emulsion stability index (ESI) (54.931 ± 1.52 min), foaming stability (87.867 ± 0.519%), oil holding capacity (OHC) (6.664 ± 0.0551 g oil/g) and foaming capacity (76.385 ± 1.251). In contrast, it displayed poor water holding capacity (WHC) (1.151 ± 0.021 g H_2O/g). These findings suggest that pine nuts protein isolate has the potential to be applied as a food ingredient in various food applications.

Keywords: *Pinus pinea* L., Protein isolates, Alkali extraction, Optimization, Functional properties, Biochemical characterization.

I.1. Materials and methods

I.1.1. Preparation of defatted pine nuts flour

Shelled and skinned pine nuts (*Pinus pinea* L.) harvested from Chrea Mountains (36.4268° N, 2.8765° E) were purchased at a local market in Blida, Algeria. The cleaned and dried pine nuts were ground to a fine powder using an electric grinder and then defatted with *n*-hexane (1:10, w/v) using a Soxhlet extractor for 6 h at 40°C. The defatted flour was air-dried overnight, sieved (200 µm), and stored in airtight containers at -20 °C until extraction.

Commercial soy protein isolate (Myprotein™, No 05016010, voyager house, Chicago Avenue, Manchester, Uk. 90% protein content) and pea protein isolate (Bulk™, Gunfleet, Colchester, CO4 9QX, UK. 80 % of protein content) were used for comparison purposes. All chemicals used were of analytical grade and purchased from either Sigma Aldrich or Biochem Chemopharma.

I.1.2. Optimization of alkali extraction conditions of protein isolates

In order to optimize the alkali extraction conditions for *Pinus pinea* L. nuts protein isolates, Response Surface Methodology (RSM) was employed. This involved the investigation of three separate factors: pH (X_1), extraction time (X_2), and solvent-to-sample ratio (X_3). These variables were selected to analyze their influence on the resulting protein isolates, employing a Box-Benhken design (BBD).

Box-Behnken designs (BBD) belong to a category of second-order designs that are rotatable or almost rotatable, derived from incomplete factorial designs with three levels. When considering three factors, their visual representation can be depicted in two ways: as a cube containing the central point and midpoints of the edges, or as a configuration of three interlocking 2^2 factorial designs along with a central point, illustrated in Figure 13.

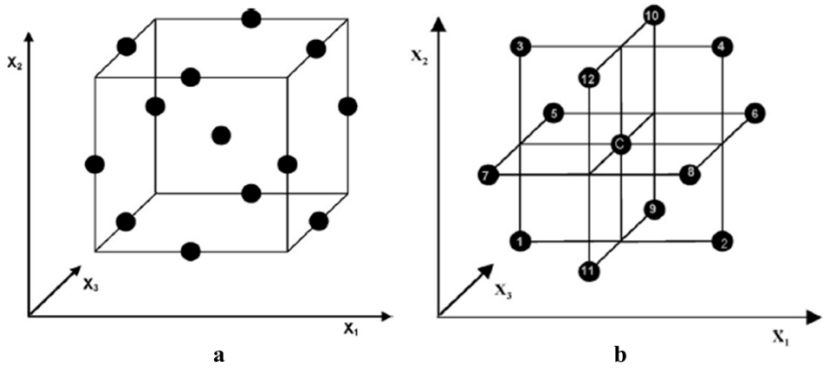

Figure 13: The cube for BBD (a) and three interlocking 2^2 factorial design (b).

The necessary quantity of experiments (*N*) for constructing a BBD is determined by the formula $N = 2k(k-1) + C_0$, where *k* represents the count of factors, and *C0* is the number of central points.

The comparison among different response surface designs (BBD, central composite, Doehlert matrix, and three-level full factorial design) indicates that both BBD and Doehlert matrix show slightly better efficiency than the central composite design and are significantly more efficient than the three-level full factorial designs. Efficiency here is defined as the ratio of coefficients in the model to the number of experiments. Another advantage of BBD is its exclusion of combinations where all factors are at their highest or lowest levels simultaneously. This characteristic is valuable for avoiding experiments conducted under extreme conditions that could lead to unsatisfactory results (Ferreira et al., 2007).

I.1.2.1. Experimental design

A Box-Behnken design was employed, encompassing a total of 15 experiments, which included 3 center points. Each individual factor was coded at three levels (-1, 0, 1), as detailed in Table V. All the experiments were carried out in a randomized sequence. The response functions chosen were protein content (g/100g), solubility (%), and emulsifying activity of the protein isolates. The experimental data were then fitted using a second-order polynomial model. This model served both to predict the optimal point and to establish correlations between the responses and the independent variables. The second-order polynomial equation adopted the following form:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{I=2}^{K} \beta_{ii} X_i^2 + \sum_{i=1}^{k=1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j \quad (1)$$

Where, **y** represents the dependent variables, that are affected by the independent variables Xi and Xj. regression coefficients β_0, β_i, βii, and βij are represented for the model's constant, linear, quadratic, and cross-product term, respectively.

The experimental design data was analyzed and the optimal conditions for each response, along with predicted responses, were estimated using the JMP 7 software package (SAS Institute Inc., Cary, NC, USA). To assess the significance of the model and the effect of the studied factors, an analysis of variance (ANOVA) was conducted. The significance of the regression coefficients

for linear, quadratic, and interactive terms was determined using an F-value with a probability of 0.05. Subsequently, three-dimensional response surface graphs were generated for each response by utilizing the regression coefficients.

Table V: Box-Behnken (BBD) experimental design of three independent variables, and their experimental and predicted responses.

run	Independent variables (factors)		
	X_1	X_2	X_3
1	9 (0)	60 (-1)	10 (-1)
2	9 (0)	60 (-1)	30 (1)
3	9 (0)	90 (0)	20 (0)
4	8 (-1)	60 (-1)	20 (0)
5	8 (-1)	120(1)	20 (0)
6	10 (1)	120(1)	20 (0)
7	10 (1)	90 (0)	30 (1)
8	10 (1)	60 (-1)	20 (0)
9	8 (-1)	90 (0)	10 (-1)
10	9 (0)	120(1)	30 (1)
11	9 (0)	120(1)	10 (-1)
12	10 (1)	90 (0)	10 (-1)
13	8 (-1)	90 (0)	30 (1)
14	9 (0)	90 (0)	20 (0)
15	9 (0)	90 (0)	20 (0)

X_1= Extraction pH, X_2 = Extraction time (min), X_3 = Sample-to-solvent ratio (g/mL).

I.1.2.2. Preparation of protein isolates

To prepare the protein isolates, the alkali extraction and isoelectric precipitation (AE/IP) method outlined by Joshi et al. (2011) was employed. Briefly, 1 g of defatted pine nut flour was dispersed in distilled water at varying solvent-to-sample ratios (1:10-1:30 g/mL), and the pH of these dispersions was adjusted to different levels (ranging from 8.0 to 12.0) using 0.1 M NaOH and stirred at room temperature for different durations (60-120 min). Afterward, the mixtures were subjected to centrifugation at 3000 rpm for 15 min and the supernatants were recovered and set aside.

The same procedure was repeated a second time using half of the initial volume to increase the extraction yields. The supernatants from the two rounds were combined and their pH was adjusted to 4.5 using 0.1 M HCl in order to precipitate proteins. The resulting mixture was centrifuged at 2500 rpm for 15 min to recover the precipitated proteins. The recovered precipitates were then re-dispersed in distilled water, neutralized with 0.1 M NaOH, and subjected to

lyophilization over a period of 24 h. The resulting protein isolates were stored at -20°C until further analysis.

I.1.3. Determination of protein content

The protein content of the pine nut protein isolates was determined using the Bradford (1976) method. Bovine serum albumin (BSA) was employed as a standard for this quantification. By utilizing the calibration curve of BSA (represented as $y = 0.5443x + 0.0332$; with an $R^2 = 0.999$), the protein content was calculated and expressed in terms of grams per 100 grams (g/100g).

I.1.4. Functional properties of *P. pinea* L. protein isolates (PPPI)

I.1.4.1. Solubility of PPPI

The solubility of PPPI was determined following the method described by Stone et al. (2015) with slight modifications. Protein isolates were prepared by dispersing 100 mg of protein in 9 mL of a 1.0 M NaCl solution to create protein suspensions. The pH of the suspensions was adjusted to 7.0 using either 0.1 M NaOH or 0.1 M HCl. After being stirred for 1 hour at room temperature, the mixtures' volume was adjusted to 10 mL, and it was allowed to sit for an additional 10 min to promote precipitation. Subsequently, the mixtures were centrifuged at 2500 rpm for 15 min. The protein content in the resulting supernatant was quantified using Bradford (1976) method, with BSA serving as a standard. Proteins solubility (%) was calculated using the following formula:

$$\text{Solubility (\%)} = P_1 / P_0 \times 100 \quad (2)$$

Where P_1 is the protein content of the supernatant and P_0 is the total protein content of the sample.

I.1.4.2. Emulsifying activity of PPPI

Emulsifying activity of PPPI was assessed following the turbidimetric method originally described by Pearce and Kinsella (1978), as described by Wani et al. (2015) with some modifications. A volume of 3 mL of protein isolate solutions, prepared at a 0.2% (w/v) in 10 mM phosphate buffer (pH 7.0), was mixed with 1 ml of sunflower oil. The mixture was homogenized using an ultraturax set at a speed of 10,000 rpm for 2 min. From the bottom of the container, 50 µL of emulsions were recovered and diluted to an appropriate concentration using 0.1% SDS

solution. The diluted solutions were vortexed for 10 seconds, and the initial absorbance (A_0) was immediately measured at 500 nm using UV-visible spectrophotometer, with 0.1% SDS solution serving as a blank.

The emulsifying activity index (EAI) was calculated using the following formula:

$$EAI\ (m^2/g) = (2 \times 2.203 \times A_0 \text{ x } F) / (L \times C \times \varphi \times 10000) \quad (3)$$

Where, A_0 represents the immediate absorbance of the diluted emulsions right after homogenization (0 min), F stands for the dilution factor, L is the cuvette's path length (1 cm), C denotes the weight of protein/volume (protein concentration in the dispersion) (g/mL) and φ represents the volumetric fraction of oil.

I.1.5. Validation of the model

The adequacy of the model was verified for each response by examining the coefficient of determination (R^2), lack of fitness, and the F-value of the model. The optimal conditions were determined using the global desirability function. This approach converts each response (Yk) into individual desirability (dk) that ranges from 0 to 1, with 0 indicating responses outside an acceptable range and 1 representing fully desirable response. To confirm the model's validity, three additional experiments were conducted under the desirable optimal conditions recommended by the model. The average results of these experiments were then compared against the model's predicted values. The optimized pine nut protein isolate, referred to as OPPPI, underwent a thorough evaluation encompassing proximate analysis, emulsion stability, foaming properties, water/oil holding capacities, and biochemical characterization.

I.1.6. Proximate Analysis

The moisture, fat and ash contents in both OPPPI and defatted pine nut flour (DPNF) were determined according to AOAC official methods: 925.09, 932.06 and 925.09, respectively (AOAC, 1990). The moisture content was determined by subjecting the samples to an oven-drying at 105°C until a constant weight was attained. Fat content was determined through solvent extraction in a Soxhlet apparatus using petroleum ether as a solvent. For ash content determination, the samples were placed into porcelain crucibles and incinerated in a muffle furnace at 550°C until a consistent weight was reached. Carbohydrate content was evaluated using the Anthrone method, employing glucose as a standard (Yemm and Willis, 1954).

I.1.7. Functional properties of OPPPI

I.1.7.1. Emulsion stability

Emulsion stability index (ESI) of OPPPI was determined at different time intervals (10, 30, 60 and 90 min) of homogenization as follows Wani et al. (2015):

$$ESI\ (min) = (A_0 \times t) / (A_0 - A_t) \quad (4)$$

Where, t represents the time interval, while A_t corresponds to the absorbance measurement of the diluted emulsion taken at 10, 30, 60 and 90 min subsequent to homogenization.

I.1.7.2. Foaming capacity and stability

The foaming capacity assessment of OPPPI followed the methodology of Agboola et al. (2005) with slight modifications. A volume of 10 mL of a 1% (w/v) protein isolate dispersion, prepared in 10 mM phosphate buffer (pH 7.0), underwent homogenization using a blender at 10,000 rpm for 3 min. The initial foam volume was immediately measured (0 min) after homogenization, as well as at 10, 30, 60, and 90 minutes, using a graduated cylinder for measurement.

Foaming capacity (FC) and foaming stability (FS) were calculated using the following formulas:

$$FC\ \% = (V_{fi} - V_i) / V_i \times 100 \quad (5)$$

$$FS\ \% = V_{ft} / V_i \times 100 \quad (6)$$

Where, V_i stands for the initial volume of protein solution prior air introduction, V_{fi} signifies the foam volume immediately after air introduction, and V_{ft} represents the remaining foam volume after a specific time interval (10, 30, 60, and 90 min).

I.1.7.3. Water and oil holding capacities

The determination of water holding capacity (WHC) and oil holding capacity (OHC) followed the procedure outlined by Wu et al. (2009). In concise, 100 mg of OPPPI were weighed and placed in pre-weighed centrifuge tubes. To these tubes, 1 mL of water or sunflower oil was added, and the samples were fully dispersed using a vortex mixer for 1 min. Subsequently, the dispersions were left to stand for 30 min at room temperature, after which they underwent centrifugation at 3000 rpm for 15 min. The resulting supernatants were carefully decanted, and

any excess of water or oil was drained. The tubes, containing the sediments, were re-weighed. WHC and OHC were expressed as the amount in grams of retained water and oil per gram of the OPPPI sample.

I.1.8. Biochemical characterization of OPPPI

I.1.8.1. Fourier-transform infrared (FTIR) spectroscopy analysis

To analyze the constituents of OPPPI and assess its secondary structure, Fourier-transform infrared (FTIR) spectroscopy (JASCO FT/IR-4200, Japan) was used. The infrared spectrum of OPPPI was recorded within the spectral range of 4000-400 cm^{-1} at room temperature, with a measurement resolution set at 8 cm^{-1}. Analysis of the resulting spectrum was carried out using Origin Pro version 8.5 software.

I.1.8.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis of OPPPI was conducted using a 12% resolving gel and a 4% stacking gel, following the method described by Laemmli (1970) with minor modifications. The analysis was carried out under both reducing conditions (in the presence of 2-mercaptoethanol) and non-reducing conditions (without 2-mercaptoethanol). The protein samples were mixed with Laemmli buffer at a 1:2 ratio (v/v) to achieve a final concentration of 1 mg/ml. Subsequently, the samples were heated at 95°C for 5 minutes, and approximately 10 µl of each sample was loaded onto the gel. Electrophoresis was performed at 70 V (15 mA) for 20 minutes, followed by 120 V (18 mA) for 100 minutes using a Tris-glycine-SDS running buffer. For staining, the gel was immersed overnight in a staining solution composed of Coomassie Blue G-250 (0.15 w/v) dissolved in a mixture of 40% methanol (v/v), 10% acetic acid (v/v), and 50% distilled water. The gel was subsequently destained using a solution containing 40% methanol, 10% acetic acid, and 50% distilled water. A pre-stained molecular weight marker (Sigma-Aldrich, Inc, St. Louis, MO, USA) was employed as a reference standard.

I.2. Results and discussion

I.2.1. Optimization of alkali extraction conditions of protein isolates

By employing a Box-Behnken design, the effect of alkali extraction conditions on extracted protein isolates was investigated. Protein content (g/100 g of extract) of the isolates as well as their solubility (%) and emulsifying activity were selected as response factors. The experimental matrix

design of 15 experiments including experimental and predicted results is shown in Table VI. The results demonstrated that the protein content, solubility, and emulsifying activity within the different tested conditions ranged from 64.752 to 97.84 g/100g of extract, 55.9 to 90.88%, and from 17.604 to 32.721 m^2/g, respectively.

Table VI: Box-Behnken (BBD) experimental design of three independent variables, and their experimental and predicted responses.

run	Independent variables (factors)			Dependent variables (responses)					
	X_1	X_2	X_3	Protein content (g/100 g)		Solubility (%)		EAI (m^2/g)	
				Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	9 (0)	60 (-1)	10 (-1)	77.73	77.590	84.97	84.280	17.783	17.393
2	9 (0)	60 (-1)	30 (1)	87.825	87.202	75.71	75.824	24.669	24.807
3	9 (0)	90 (0)	20 (0)	97.84	96.931	72.81	71.163	20.699	20.253
4	8 (-1)	60 (-1)	20 (0)	86.51	87.451	88.22	88.663	22.661	22.341
5	8 (-1)	120(1)	20 (0)	85.25	85.429	83.46	83.328	28.796	28.233
6	10 (1)	120(1)	20 (0)	86.59	85.651	76.19	75.746	17.604	17.922
7	10 (1)	90 (0)	30 (1)	85.49	86.291	64.035	63.788	26.346	25.635
8	10 (1)	60 (-1)	20 (0)	64.752	64.573	55.9	56.031	23.748	24.296
9	8 (-1)	90 (0)	10 (-1)	91.32	90.518	90.88	91.126	24.706	24.410
10	9 (0)	120(1)	30 (1)	94.08	94.219	83.55	84.239	21.169	25.410
11	9 (0)	120(1)	10 (-1)	89.006	89.628	90.36	90.245	20.311	20.162
12	10 (1)	90 (0)	10 (-1)	71.82	72.138	61.5	62.057	24.485	24.309
13	8 (-1)	90 (0)	30 (1)	90.88	90.568	75.49	74.932	32.721	32.890
14	9 (0)	90 (0)	20 (0)	96.34	96.931	70.32	71.163	20.008	20.253
15	9 (0)	90 (0)	20 (0)	96.615	96.931	70.36	71.163	20.063	20.253

X_1= Extraction pH, X_2 = Extraction time (min), X_3 = Sample-to-solvent ratio (g/ml), EAI = Emulsifying activity index (m^2/g).

I.2.1.1 Model Fitting

An essential step in assessing the model's significance involves performing Analysis of Variance (ANOVA) to determine its validity. The models displayed a high level of significance ($p < 0.0001$) for protein content and solubility, with respective F-values of 122.776 and 142.570. The coefficient of determination, R^2, was 0.995 for protein content and 0.996 for solubility. A comparison between R^2 and R^2adj values revealed negligible differences, implying a strong correlation between experimental and predicted data. Likewise, the model's significance was established ($p = 0.0002$) for emulsifying activity, supported by an F-value of 52.179, as outlined in Table VII. The coefficient of determination, R^2, for emulsifying activity (0.989) closely

resembled R^2adj (0.970). In all models, the non-significance of the lack of fit further confirms the model's validity. These results underline the model's suitability and good fitness.

The precision and reproducibility of the model, indicated by the Coefficient of Variation (CV), is an important metric. Its value, ideally below 10%, was calculated to be 1.19%, 1.46%, and 3.06% for protein content, solubility, and emulsifying activity, respectively. These CV values underline the experiments' reliability and accuracy.

Table VII: Regression coefficients (β) estimate and analysis of variance (ANOVA) for the response surface quadratic models of protein content (Y_1), solubility (Y_2), and emulsifying activity (Y_3) of PPPI.

Source	Sum of squares	Degree of freedom (DF)	Regression coefficients (β)	P-value	F-value
Protein Content (g/100g of extract)					
Model	1196.885	9	96.931	<,0001	122.776
Linear					
X_1	256.647	1	-5.664	<,0.001	256.647
X_2	181.565	1	4.764	<,0.001	167.624
X_3	100.862	1	3.550	<,0.001	93.118
Quadratic					
X_1^2	313.755	1	-9.218	<,0.001	289.664
X_2^2	177.691	1	-6.937	<,0.001	164.048
X_3^2	29.659	1	-2.834	0.0034	27.382
Cross product					
X_1X_2	133.414	1	5.775	0.0001	123.170
X_1X_3	49.723	1	3.525	0.0011	45.905
X_2X_3	6.302	1	-1.255	0.0607	6.302
Lack of fit	4.140	3		0.3316	2.164
Pure error	1.275	2			
Total error	5.415	5			
Cor total	1202.301	14			
R^2	0.9954				
R^2 adj	0.9873				
CV%	1.19				
Solubility (%)					

Model	1594.961	3	71.163	<,0.001	142.570
Linear					
X_1	808.522	1	-10.053	<,0.001	650.450
X_2	103.392	1	3.595	0.0003	83178
X_3	104.581	1	-3.615	0.0003	84.135
Quadratic					
X_1^2	32.046	1	-2.946	0.0038	25.780
X_2^2	220.352	1	7.725	<,0.001	177.272
X_3^2	83.622	1	4.758	0.0004	67.273
Cross product					
X_1X_2	156.875	1	6.262	<,0.001	126.205
X_1X_3	80.326	1	4.481	0.0005	64.622
X_2X_3	1.500	1	0.612	0.321	1.207
Lack of fit	2.147	3		0.797	0.351
Pure error	4.068	2			
Total error	6.215	5			
Cor total	1601.1770	14			
R^2	0.9961				
R^2 adj	0.9891				
CV%	1.46				
			Emulsifying activity (m^2/g)		
Model	235.009	9	20.253	0.0002	52.179
Linear					
X_1	34.911	1	-2.089	0.0004	69.763
X_2	0.116	1	-0.120	0.6493	0.233
X_3	38.777	1	2.201	0.0003	77.488
Quadratic					
X_1^2	75.211	1	4.513	<,0001	75.211
X_2^2	9.083	1	-1.568	0.0080	9.083
X_3^2	19.439	1	2.294	0.0016	19.439
Cross product					
X_1X_2	37.613	1	-3.066	0.0003	37.613
X_1X_3	9.467	1	-1.538	0.0003	9.467
X_2X_3	9.063	1	-1.505	0.0074	9.063
Lack of fit	2.215	3		0.1671	5.143
Pure error	0.287	2			
Total error	2.502	5			
Cor total	237.511	14			
R^2	0.9894				
R^2 adj	0.9705				
CV%	3.06				

I.2.1.2. Analysis of surface responses

I.2.1.2.1. Protein content (PC)

All factors exhibited linear, quadratic, and interactive influences on the protein content of the isolates, as shown in Table VII. In terms of linear effects, extraction pH, extraction time, and solvent-to-sample ratio displayed remarkable significance ($p < 0.001$). Similarly, the quadratic impact of pH and extraction time demonstrated high significance ($p < 0.001$), followed by the quadratic effect of solvent-to-sample ratio, which was also notable ($p = 0.003$). Among the interactive terms, both the interaction between pH and extraction time ($p = 0.0001$) and the interaction between pH and solvent-to-sample ratio ($p = 0.0011$) held significance. However, the interaction between extraction time and solvent-to-sample ratio did not show significance ($p > 0.05$). Upon excluding non-significant variables, the second-order polynomial equation that best represented the protein content response was derived as follows:

$$Y_{PC} = 96.931 - 5.664\ X_1 + 4.764\ X_2 + 3.550\ X_3 - 9.218\ X_1^2 - 6.937\ X_2^2 - 2.834\ X_3^2 + 5.775\ X_1X_2 + 3.525\ X_1X_3 \quad (7)$$

Protein isolates represent highly concentrated fractions (containing over 85% protein on a wet basis), rendering them purer than protein concentrates. Numerous food applications require protein isolates of high purity, coupled with favorable yield, thus highlighting the significance of protein content as a crucial attribute for these isolates (Sandberg, 2011; Aluko, 2017; Mir et al., 2019b).

The highest recorded PC (97.84 g/100g of extract) was observed when the extraction was performed at pH 9, for a duration of 90 min, and using a solvent-to-sample ratio of 1:20 g/ml. According to Equation (7), the pH of extraction displayed significant negative effects for both linear and quadratic terms. As shown in Figure 14.a, the protein content reached its peak at pH 9. An increase in pH from 8 to 9 resulted in a slight rise in protein content, indicating the high protein extractability in alkaline mediums (Liu et al., 2013). The alkaline medium causes proteins to become negatively charged due to ionization of carboxylic groups and deprotonation of amine groups. This electrostatic repulsion between similarly charged proteins enhances protein solubility (Mir et al., 2019b).

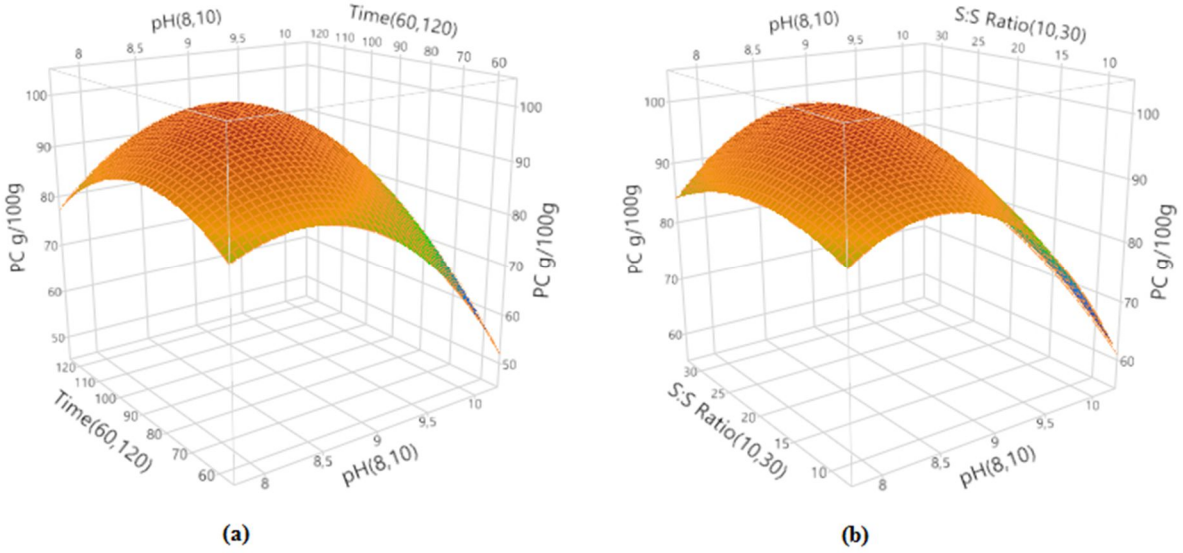

Figure 14: 3D response surface graphs showing interactive effects of pH and time (a) and pH and solvent-to-sample ratio (b) on the protein content of protein isolates.

However, a further pH increase (above 9) led to a substantial reduction in PC, reaching a purity level of approximately 54 g/100g at pH 10. Similar findings were reported by Mir et al. (2019b) and Ruiz et al. (2016) who also noted that elevated extraction pH correlated with diminished PC in album and quinoa protein isolates. The decline in PC could be attributed to the presence of non-protein components in highly alkaline condition, co-precipitating with proteins and subsequently lowering isolate purity (Ruiz et al., 2016; Feyzi et al., 2018). Despite the decrease in PC associated with extremely alkaline mediums, other studies have reported that extraction yield increases with increasing extraction pH (Firatligil-Durmus and Evranuz, 2010; Siow and Gan, 2014; Garg et al., 2020).

The extraction time had a significant positive influence on the PC of the isolates. As the extraction time increased, the PC exhibited a gradual increase until it peaked at 100 min, after which a decline was observed. The gradual rise in PC with prolonged extraction time can be attributed to the extended period allowing a greater diffusion of proteins from the flour into the solution. However, the subsequent decrease in PC beyond 100 min may indicate the attainment of extraction equilibrium, suggesting that the maximum amount of proteins had been extracted (Lestari et al., 2010). Nevertheless, Feyzi et al. (2018) reported differing results, noting that an increase in extraction time beyond 50 min led to an increase in protein content for grass pea protein

isolates, followed by a decrease with further extension. Similarly, Garg et al. (2020) found that protein solubilization reached saturation around 120 min of extraction.

Moreover, increasing the extraction time from 60 to 90 min in conjunction with increasing the pH from 8 to 9 resulted in a significant and substantial increase in the PC of the isolates. This notable increase in PC could be attributed to subjecting the defatted flour to prolonged alkalization, which facilitated a high solubilization of proteins. However, once the extraction surpassed pH 9 and 90 min, a decline in PC was observed. This reduction could be linked to protein denaturation caused by the extreme alkaline nature and polarity of the medium (Du et al., 2018).

The solvent-to-sample ratio had a significant impact on PC. Increasing the water volume from 10 to 15 mL led to a slight increase in PC (~ 85 g/100g), which remained relatively stable until a solvent-to-sample ratio of 1:25 g/mL, after which it started to decline (figure 14.b). According to Liadakis et al. (1995), higher solvent-to-sample ratios facilitate protein diffusion and extraction from flour to the solution. Common extraction methods employ ratios between 1:5 and 1:30 g/mL, often with repeated extractions, as this approach using small water volumes and multiple extractions is more efficient (Aguilera and GARCIA, 1989; Kain et al., 2009). Additionally, smaller solvent volumes are preferable on an industrial scale due to ease of handling and compatibility with smaller equipment such as mixers and decanters.

Furthermore, both an increase in pH and solvent-to-sample ratio significantly increased the PC of the isolates. However, the interactive effect of extraction time and solvent-to-sample ratio did not exert a significant influence on the PC.

I.2.1.2.2. Solubility (SOL)

As depicted in Table VII, it is evident that the solubility of protein isolates was primarily influenced by two key factors: the extraction pH (X_1) and the extraction time (X_2). The linear impact of pH, the quadratic influence of time, and the interactive effect of pH and time were highly significant ($p < 0.0001$). Furthermore, both the linear effects of time and solvent-to-sample ratio displayed considerable significance ($p = 0.0003$), followed by the quadratic effects of solvent-to-sample ratio ($p = 0.0004$) and pH ($p = 0.0038$). Additionally, the interactive term between pH and solvent-to-sample ratio were significant ($p = 0.0005$), although the interaction between time and solvent-to-sample ratio did not show significance ($p > 0.05$). The final second-order polynomial equation for solubility, after excluding variables that were not significant, is represented as follows:

$$Y_{SOL} = 71.163 - 10.053\ X_1 + 3.595\ X_2 - 3.615\ X_3 - 2.946\ X_1^2 + 7.725\ X_2^2 + 4.758\ X_3^2 + 6.262\ X_1X_2 + 4.481\ X_1X_2 \quad (8)$$

Solubility is an essential prerequisite for using proteins in various moisture-rich food applications and stands as a vital functional property, which can significantly influence other functional properties (Adenekan et al., 2018; Gao et al., 2018). As proteins possess amphoteric polyelectrolyte characteristics, factors such as pH and the ionic strength of aqueous solvents can have a significant effect on their solubility (Li et al., 2018). In this study, the protein isolates were solubilized in a 1.0 M NaCl solution (pH 7.0) due to their higher solubility in saline solutions compared to distilled water and other aqueous media. This high solubility in saline solutions for *P. pinea* L. nuts protein isolates is likely attributed to the presence of globulins. Nasri and Triki (2007) highlighted that globulin constituted the predominant fraction, making up 74.74% of the total storage proteins in *Pinus pinea* L. pine nuts.

From Table VII and Equation 8, it is evident that the solubility of the isolates was significantly influenced by pH and extraction time. The highest SOL value (90.88%) was recorded for the protein isolate obtained at extraction pH 8, an extraction time of 90 min, and a solvent-to-sample ratio of 1:10 g/mL. Stone et al. (2015) reported notably lower SOL for pea protein isolates (62.7-64.4%), obtained under different extraction conditions (pH 9.5, time 60 minutes, and solvent-to-sample ratio of 1:15 g/mL). In comparison, the commercial soy protein isolate (SPI) and pea protein isolate (PPI) tested in this study exhibited even lower SOL than the pine nut protein isolates. Specifically, SPI displayed a solubility of 20.59 ± 0.289%, while PPI exhibited a solubility of 10.34 ± 0.132%.

The linear impact of pH on protein solubility was highly significant. As shown in Figure 15a, increasing the extraction pH from 8 to 10 resulted in a notable decrease in the solubility of the isolates, with the highest solubility values observed for proteins obtained at pH 8. This decrease in solubility suggests a reduction in protein-solvent interactions and an induction of hydrophobic interactions between proteins. The decrease in solubility due to higher alkaline extraction pH could be attributed to conformational changes that proteins underwent during the alkaline extraction process.

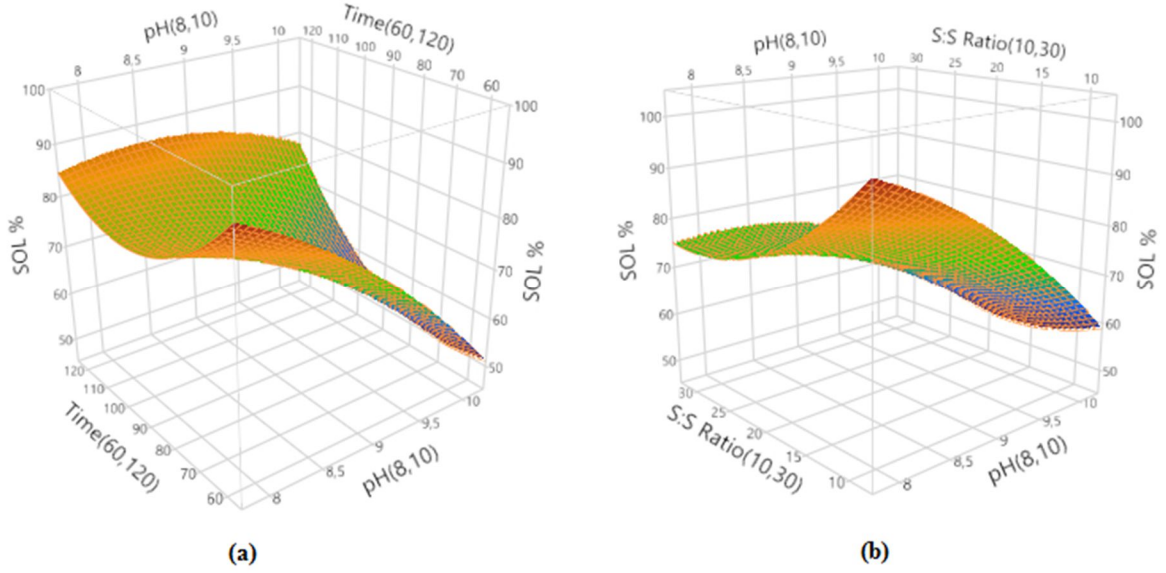

Figure 15: 3D response surface graphs showing interactive effects of pH and time (a) and pH and solvent-to-sample ratio (b) on the solubility of protein isolates.

The solubility of proteins is closely associated to their folding and the exposure of hydrophilic/hydrophobic groups. High alkaline pH levels might lead to the unfolding of proteins, causing the exposure of hydrophobic groups. This exposure can trigger protein-protein interactions and subsequent protein aggregation, resulting in decreased solubility. The variations in protein solubility at different pH levels are probably due to the extent of denaturation. Higher alkalinity corresponds to more pronounced unfolding of proteins. In contrast, the high solubility of proteins at lower alkaline pH suggests lesser denaturation and a more native protein state (Krause et al., 2002; Joshi et al., 2011; Liu et al., 2011; Stone et al., 2015; López et al., 2018b; Mir et al., 2019b).

Furthermore, it has been reported that the extraction method can influence the conformation of proteins (Mwasaru et al., 1999). Alkaline extraction and isoelectric precipitation, when compared to alternative extraction methods, have been demonstrated to yield proteins with reduced solubility and high surface hydrophobicity (Karaca et al., 2011b; Stone et al., 2015). Additionally, an inverse relationship between solubility and surface hydrophobicity has been observed in protein isolates from faba beans, lentils, peas, and beans (Karaca et al., 2011b). The high surface hydrophobicity of proteins can be attributed to the fact that this particular extraction method predominantly extracts globulins, which are known to exhibit greater surface hydrophobicity in comparison to albumins (Papalamprou et al., 2009). Moreover, Mwasaru et al. (1999) reported an

increase in the hydrophobicity value of pigeon pea protein isolate with an increase of extraction pH to 10.5.

Time exerted positive significant linear and quadratic influences on protein solubility. Similar to pH, shorter time periods (60 min) led to higher solubility for the isolates. With an extension to 80 min, there was a slight reduction in solubility. Beyond this point, protein SOL reached a plateau ~75%, implying a limited unfolding of proteins. Furthermore, the interactive term of time and pH had a positive and significant impact on solubility. As time and pH increased, protein SOL demonstrated an increase. Nevertheless, this increase was less pronounced compared to when pH and time were at their minimum levels (pH 8 and time 60 min).

Similar surface patterns were observed for both pH and solvent-to-sample ratio (Figure 15b). Solvent-to-sample ratio exhibited a significant negative effect on isolates solubility; increasing the water volume from 10 to 20 mL corresponded to a decrease in isolates solubility, beyond which no further reduction was observed when the volume was increased to 30 mL. Additionally, the interactive effect of solvent-to-sample ratio and time was not significant. In contrast, the interactive effect of solvent-to-sample ratio and pH significantly affected solubility, with an increase in both variables leading to a slight increase in protein isolate solubility. This increase in protein solubility with increasing pH and solvent-to-sample ratio (X_1X_3) as well as pH and time (X_1X_2) could be attributed to the occurrence of more protein-solvent interactions, thereby enhancing protein solubility.

I.2.1.2.3. Emulsifying activity (EA)

The emulsifying activity of proteins was significantly influenced by linear, quadratic, and interactive terms, except for the linear term of time (X_2). From Table VII, it has been observed that the quadratic term of pH $(X_1)^2$ had a great impact on protein emulsifying activity, exhibiting a remarkably significant effect ($p < 0.0001$) compared to the interactive terms of pH and time (X_1X_2) and pH and solvent-to-sample ratio (X_1X_3) ($p = 0.0003$). The linear term of solvent-to-sample ratio exhibited a highly significant effect ($p = 0.0003$), followed by pH ($p = 0.0004$), whereas the linear term of time was not significant ($p > 0.05$). Additionally, for the quadratic terms, both time $(X_2)^2$ ($p = 0.0080$) and solvent-to-sample ratio $(X_3)^2$ ($p = 0.0016$) had significant effects on emulsifying activity. Similarly, the interactive effect of time and solvent-to-sample ratio (X_2X_3) was also significant ($p = 0.0074$). The final second-order polynomial equation for emulsifying activity, after excluding non-significant factors, can be formulated as follows:

$$Y_{EAI} = 20.053 - 2.089\ X_1 + 2.201\ X_3 + 4.513\ X_1^2 - 1.568\ X_2^2 + 2.294\ X_3^2 - 3.066\ X_1X_2 - 1.538\ X_1X_3 - 1.505\ X_2X_3 \quad (9)$$

The determination of protein isolate emulsifying activity involved assessing the emulsifying activity index (EAI), a metric that estimates the surface area at the interface that can be effectively stabilized by a unit weight of protein (Lam et al., 2018). Emulsifying activity is defined as to the capacity of proteins to interact with both water and oil, facilitating the creation of emulsions, which is influenced notably by factors such as the ionic charge and hydrophobic properties of proteins' surface (Zhang et al., 2020).

In the current study, the protein isolates exhibited varying emulsifying activities. The lowest EAI value (17.604 m^2/g) was observed when the extraction was carried out at pH 10, for a duration of 120 min, and with a solvent-to-sample ratio of 1:20 g/mL. On the other hand, the highest EAI value (32.721 m^2/g) was achieved when the protein extraction was performed at pH 8, for 90 min, and with a solvent-to-sample ratio of 1:30 g/mL. These results are higher than the emulsifying activity indices reported for kidney bean protein isolates (15.8-26.6 m^2/g) and field pea protein isolates (11.8-14.1 m^2/g) as reported by Shevkani et al. (2015). However, they are lower than the EAI observed for flaxseed concentrate (87.1 m^2/g) according to Tirgar et al. (2017). In comparison to commercially available soy and pea protein isolates, both proteins demonstrated emulsifying activity indices of 30.70 m^2/g and 26.7 m^2/g for soy and pea protein isolates, respectively, which were comparable to the highest values discovered in this study.

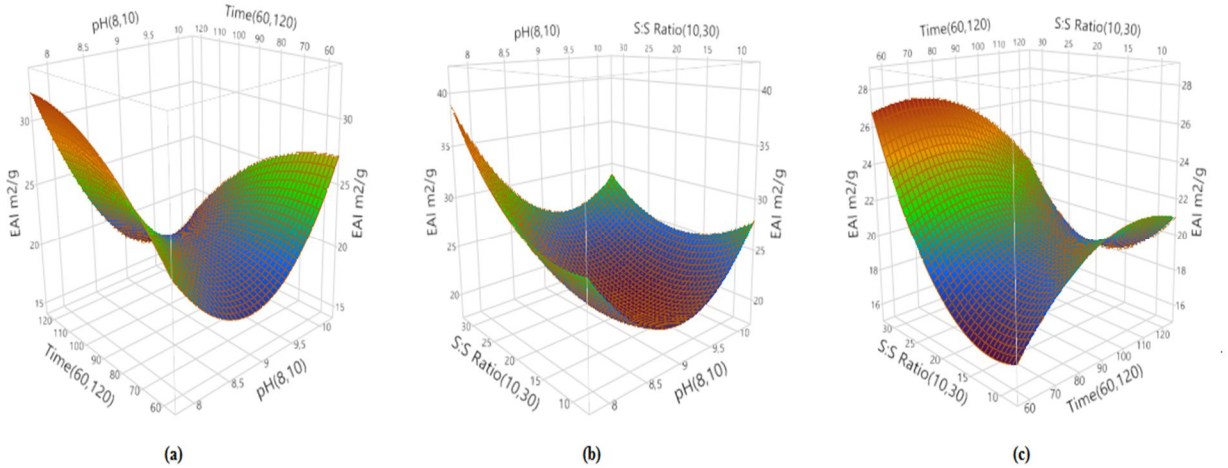

Figure 16: 3D response surface graphs showing interactive effects of pH and time (a), pH and solvent-to-sample ratio (b) and time and solvent-to-sample ratio (c) on the emulsifying activity of protein isolates.

Numerous factors can influence the emulsifying activity of proteins. Nonetheless, protein solubility and surface hydrophobicity have been identified as the predominant elements having a substantial impact on protein emulsifying properties (Liu et al., 2011). The effect of extraction pH on emulsifying activity is shown in Figure 16a. The extraction pH exhibited a significant negative linear influence on isolate emulsifying activity. The EAI gradually decreased as extraction pH increased from 8 to 9, followed by a sharp increase, culminating in an EAI of approximately 27 m^2/g for isolates obtained at pH 10.

Proteins obtained at pH 8 demonstrated superior emulsifying activity compared to those acquired at pH 9, largely attributed to the elevated solubility of proteins at pH 8. Enhanced solubility is conducive to effective emulsification as it facilitates the mobility and dispersion of protein molecules toward the oil-water interface, promoting their rearrangement and subsequently augmenting emulsion capacity (Dapčević-Hadnađev et al., 2019; Cai et al., 2020b).

Proteins obtained at pH 10 exhibited good emulsifying activity, possibly probably due to increased surface hydrophobicity induced by protein unfolding at highly alkaline extraction pH. This unfolding exposes hydrophobic groups previously buried within the protein core, thus contributing to increased surface hydrophobicity. Surface hydrophobicity plays an essential role in emulsion systems, impacting protein adsorption at the oil-water interface through hydrophobic interactions between oil and hydrophobic patches. Furthermore, increased surface hydrophobicity promotes protein adsorption on the oil side of the interface (Cheung et al., 2015; Nham Tran et al., 2020).

Conversely, a decrease in EAI was observed for proteins obtained at pH 9, potentially attributed to their decreased solubility and heightened surface hydrophobicity, as indicated in section I.2.1.2.2. according to Karaca et al. (2011b), the sole influence of solubility and surface hydrophobicity were positively noted, yet their interaction exhibited a negative influence on protein emulsifying activity. Moreover, authors have highlighted that the equilibrium between solubility and surface hydrophobicity significantly affects emulsifying activity (Afizah and Rizvi, 2014). Hence, the balance between hydrophilic and hydrophobic characteristics is important for emulsifying activity. This dependence is rooted in the hydrophilic-hydrophobic ratio of proteins, driving the re-orientation of hydrophobic residues towards the oil phase and hydrophilic residues towards the aqueous phase at the oil-water interface (Mohan and Mellem, 2020b).

Time exhibited no significant effect on protein EAI, while the interactive effect of pH and time demonstrated a significant negative influence. Increasing both pH and time resulted in a significant reduction in EAI, reaching approximately 17 m^2/g (Figure 16b). This reduction could be attributed to the extensive protein denaturation caused by prolonged exposure to high alkaline pH. Despite the fact that partial protein denaturation leads to soluble, aggregated proteins with increased surface hydrophobicity, thereby promoting protein adsorption at the oil-water interface, extensive and irreversible aggregation of insoluble proteins hinder their efficient unfolding at the interface. Consequently, the formation of effective films around oil droplets is impeded, leading to a decrease in emulsifying properties (Afizah and Rizvi, 2014; Yu et al., 2017).

The emulsifying activity of proteins was significantly influenced by the solvent-to-sample ratio. Increasing the water volume from 10 to 30 mL led to a noticeable increase in EAI. However, the interactive effect of pH and solvent-to-sample ratio had an adverse effect on EAI; increasing both factors resulted in a considerable decrease in EAI, possibly related to a reduction in solubility. Similarily, the interaction between time and solvent-to-sample ratio had a significant negative effect on EAI. When both variables were increased, the EAI decreased to around 22 m^2/g, while decreasing both variables resulted in a lower EAI of approximately 17.5 m^2/g. Furthermore, the lowest EAI was observed at a solvent-to-sample ratio of 1:20 g/mL.

It has been proposed that the final protein content may also impact protein emulsifying properties (Arise et al., 2015; Kaushik et al., 2016). Nonetheless, this suggestion does not fully explain the lower EAI, as a single factor cannot predict protein emulsifying activity. Various factors such as molecular size, droplet size, interfacial tension, protein conformation, net charge of the lipophilic-hydrophilic interface, pH, and ionic strength have all been reported to influence protein emulsifying properties (Bora and Ribeiro, 2004; Ragab et al., 2004; Cano-Medina et al., 2011; Wani et al., 2015). Consequently, achieving enhanced emulsifying properties necessitates finding a balance between couteracting physicochemical characteristics of proteins and their careful manipulation (Afizah and Rizvi, 2014).

I.2.1.3. Validation of the model

In order to validate the accuracy and feasibility of the model, a comparison was conducted between predicted values and experimental results obtained under predictive optimal conditions. The model suggested optimal conditions for PC involving pH 8.86, a time of 97.25 min, and a

sample-to-solvent ratio of 1:24.86 g/mL, predicting a PC of 98.76 g/100 g of extract. For Solubility, the model suggested pH 8, a time of 60 min, and a solvent-to-sample ratio of 1:10 g/mL as the optimal conditions, aiming for a predicted response of 102.1%. Similarly, for emulsifying Activity, the model suggested pH 8, a time of 103 min, and a sample-to-solvent ratio of 1:30 g/mL, aiming for a predicted EAI value of 33.22 m^2/g.

However, the primary objective of this study was to identify a common desirable optimal condition for the three responses (PC, Solubility, EAI). To achieve this, the desirability approach was employed, leading to the estimation of optimal conditions for the alkaline extraction and isoelectric precipitation. The model indicated that the optimal conditions with a desirability of 0.71 were: pH 8, a time of 107.58 min, and a solvent-to-sample ratio of 1:10 g/mL. This configuration was projected to yield an optimum protein content of 88.27 g/100g of extract, a solubility of 91.85%, and an EAI of 27.48 m^2/g.

Importantly, no statistically significant difference ($p > 0.05$) was observed between the predicted optimal values and the experimental results (protein content: 89.13 g/100g of extract, solubility: 91.16%, and EAI: 28.14 m^2/g) (Table VIII) achieved under the adjusted conditions (pH: 8, time: 108 minutes, solvent-to-sample ratio: 1:10 g/mL). As a result, it can be concluded that the model is both accurate and well-suited for the extraction of protein isolates from *Pinus pinea* L. pine nuts.

Table VIII: Predicted and experimental values of dependent variables under desirable optimal conditions.

	Dependent variables (responses)		
	Protein content (g/100g)	Solubility (%)	EAI (m^2/g)
Predicted values	88.278	91.857	27.480
Experimental values	89.138 ± 0.688	91.169 ± 0.496	28.141 ± 0.784

Results are expressed as the mean of three experiments ± SD.

I.2.2. Yield and proximate analysis of OPPPI and DPNF

Using the desirable optimal conditions in the extraction of protein isolate resulted in a yield 18.023 ± 0.767% of protein on a dry weight basis. This value was higher than those reported by Stone et al. (2015) for various cultivars of pea protein isolates (15.3-16%), and fell within the range of native kidney bean protein isolates (16.89-20.50%) reported by Wani et al. (2015), who employed the same extraction method.

The proximate composition DPNF and OPPPI is detailed in Table IX. The protein content of OPPPI measured at 89.138 ± 0.668 g/100g. This aligns with the protein content recorded for great northern bean protein isolate (89.25g/100g) (Rui et al., 2011), and is close to the protein content of pea protein isolate (88.2 g/100g) (Karaca et al., 2011b) and quinoa protein isolate (88 g/100g) (Ruiz et al., 2016). The protein content of OPPPI fulfills the criteria for high protein content typical of commercial protein isolates (Li et al., 2018).

DPNF exhibited a notable protein content of 65.368 g/100g, highlighting its impressive nutritional quality and potential as a valuable plant-based protein source. Comparable protein content was observed for defatted flour derived from *Pinus maximartinezii* pine nuts (65.6 g/100g) as reported by (Mata, 2001). In contrast, lower protein contents were reported by Wu et al. (2009) and Yu et al. (2007) for defatted peanut flour, accounting for 55.88 g/100g and 56.75 g/100g, respectively.

Despite the extensive *n*-hexane defatting, complete fat removal was not achieved for DPNF. Its fat content was 2.5%, whereas traces of fat were the only remnants in OPPPI. Similarly, the carbohydrate content decreased from 13.027% (in DPNF) to 1.913 ± 0.034 in OPPPI, indicating that minor quantities of non-protein substances were recovered at an extraction pH of 8. Moreover, the extraction process led to reductions in ash and moisture contents. As is often the case with minerals, they tend to be discarded in the supernatant after precipitation supernatant (Yu et al., 2007). The moisture content decreased from 10.843% to 3.535%, while the ash content decreased from 8.309% to 4.863 ± 0.053% in OPPPI.

Table IX: Proximate chemical composition of defatted pine nuts flour (DPNF) and optimized pine nuts protein isolate (OPPPI).

	Components (g/100g)				
	Protein	Moisture	Fat	Ash	carbohydrate
OPPPI	89.138±0.668	3.535 ±	-	4.863 ± 0.053	1.913 ± 0.034
DPNF	65.368±0,485	10.843±0.298	2.5±0.141	8.309±0.218	13.027±1.747

Results are expressed as the mean of three experiments ± SD. DPNF: Defatted pine nut flour, OPPPI: Optimized *Pinus pinea* L. protein isolate,.

I.2.3. Emulsion stability (ES) of OPPPI

Emulsion stability refers to the capacity of proteins to withstand destabilizing changes, such as creaming, flocculation, and coalescence, within a defined timeframe (Afizah and Rizvi, 2014; Gong et al., 2016).

The stability of emulsions is presented in Table X. After standing for 10 min at room temperature, the Emulsion Stability Index (ESI) of OPPPI measured 54.931 ± 1.52 min. This value was comparable with the ESI of okara protein (54 min) (Cai et al., 2020b) and exceeded those recorded for peanut protein isolate at pH 6 (19.18 min)) (Wu et al., 2009), pumpkin protein isolate (23.65 min) (Vinayashree and Vasu, 2021), and flaxseed protein isolate (12.51 min) (Tirgar et al., 2017). However, it was lower than that of the bitter melon protein isolate (63 min) (Horax et al., 2011)..

In comparison to commercial soy and pea protein isolates, the Emulsion Stability Index was 30.997 ± 0.818 min for SPI and 29 ± 0.344 min for PPI after 10 minutes of standing, both of which were inferior to OPPPI's.

Table X: Emulsion stability of OPPPI, SPI, and PPI over time.

ESI (min)	Time (min)			
	10	30	60	90
OPPPI	54.931 ± 1.152	121.854 ±1.625	182.035 ± 1.189	235.054 ± 1.613
SPI	30.997 ± 0.818	81.445 ± 1.076	129.843 ± 1.709	190.839 ± 0.00
PPI	29.175 ± 0.344	61.225 ± 0.750	108.796 ± 0.274	150.33 ± 0.761

Results are expressed as the mean of three experiments ± SD. ESI: Emulsion stability index, OPPPI: Optimized *Pinus pinea* L. protein isolate, PPI: Pea protein isolate, SPI: Soy protein isolate.

The stability of OPPPI can be attributed to electrostatic repulsions occurring between the adsorbed proteins at the interfacial film. This is facilitated by the surface charge of proteins and provides protection against coalescence and flocculation. The force of electrostatic repulsions greatly influences emulsion stability, particularly in the face of these two phenomena (Shevkani et al., 2015; Wani et al., 2015). Surface hydrophobicity has also been reported in contributing to emulsion stability, although to a certain extent (Cai et al., 2020b; Jiang et al., 2020b). Hydrophobic interactions induce protein-protein interactions, fostering the formation of strong interfacial films (Cai et al., 2020b; Jiang et al., 2020b). However, excessive exposure of hydrophobic groups can lead to irreversible protein aggregation, thereby compromising the ability to reduce interfacial tension and consequently reducing emulsion stability (Afizah and Rizvi, 2014; Cai et al., 2020b)..

Over time, a considerable decrease in ES was observed, suggesting a weakening of the interfacial film due to decreased protein-protein interactions. This weakening results in unfavorable interactions among oil droplets, leading to coalescence and larger droplet formation.

This, in turn, decreases the contact area between the two phases, causing separation and emulsion coalescence (Weiss, 2002; Mundi and Aluko, 2012).

Like OPPPI, the ES of commercial SPI and PPI also decreased with time, and their ES remained inferior to that of OPPPI even after an extended period (90 min). Overall, OPPPI demonstrated superior emulsion stability compared to commercial soy and pea protein isolates.

I.2.3. Foaming properties of OPPPI

I.2.3.1. Foaming Capacity (FC)

The Foaming Capacity of proteins is determined by their ability to undergo unfolding in specific conditions, including temperature, pH, and concentration, leading to the formation of a cohesive layer enveloping air and resulting in foam formation (Jiang et al., 2020b; Mohan and Mellem, 2020b). OPPPI demonstrated a foaming capacity of 76.385 ± 1.251% at a neutral pH (Table XI). Comparable results were recorded for the FC of the globulin protein fraction from kidney beans (76%) (Mundi and Aluko, 2012) and cashew nut shell protein isolates (76.88%) at pH 7 (Yuliana et al., 2014). In contrast, decreased FC values were observed for cashew nut protein isolate (45%) (Ogunwolu et al., 2009), *torreya grandis* seeds (24.67%) (Yu et al., 2017), and okara protein (23%) (Cai et al., 2020b).

The enhanced foaming capacity of OPPPI can be attributed to its heightened solubility, as this quality is directly linked to the protein's foaming characteristics. Effective foaming necessitates higher solubility, enabling proteins to easily and rapidely migrate to the water-air interface. This migration reduces interfacial tension and triggers conformational changes such as unfolding, facilitating interactions that lead to in the formation of a protective membrane that entraps air particles particles (Kaur and Singh, 2007; Wani et al., 2015; Jain and Anal, 2016; Li et al., 2018).

Rapid adsorption and conformational shifts in proteins, their rearrangement at the interface, and the creation of a viscoelastic cohesive film through intermolecular interactions are fundamental requisites for achieving a strong foaming capacity (Fidantsi and Doxastakis, 2001; Moure et al., 2006). Similar to emulsion capacity, FC is also influenced by pH (Shevkani et al., 2015). Researchers have reported that better FC is observed at extremely acidic and basic pH levels. For instance, Liu et al. (2013) revealed an FC of 153% at pH 3. At highly alkaline pH, Yuliana et al. (2014) recorded an FC of 90.01% at pH 10 for cashew nut shell protein isolate, and

Ragab et al. (2004) reported an FC of 92% at the same pH for cowpea proteins. The enhanced foaming capacity at extremely acidic and basic pH values can be attributed to an increase in net charge, which weakens hydrophobic interactions and enhances protein flexibility. Consequently, this facilitates increased protein diffusion to the air-water interface, allowing for the entrapment of air bubbles and resulting in improved foaming capacity (Ragab et al., 2004; Yuliana et al., 2014).

In comparison to commercial protein isolates, soy protein isolates exhibited higher foaming capacity (119.5 ± 2.12 %), surpassing that of OPPPI. On the other hand, the foaming capacity of pea protein isolate was lower than that of OPPPI (58 ± 2.828%).

I.2.3.2. Foaming Stability (FS)

Foam Stability refers to the capacity of proteins to uphold the volume of foam over a designated period of time. For foam stability, the protein films formed during the aeration process surrounding air bubbles necessitate cohesion, viscosity, and elasticity, alongside continuous air-impermeability, these conditions are all essential for foam endurance (Sai-Ut et al., 2009; Mundi and Aluko, 2012). After a 10-min period at room temperature, OPPPI displayed a foam stability of 87.867 ± 0.519% (Table XI). The high FS of OPPPI can be attributed to the strength of the interfacial film formed by adsorbed proteins. This result stemmed from a great protein-protein interactions at the interface, resulting in increased viscosity due to augmented interactions between water and protein molecules. As a result, this facilitated the formation of a coherent multilayer film (Zhang et al., 2020). Furthermore, a higher protein content was correlated with improved foam stability, as it fostered enhanced protein-protein interactions and increased viscosity, thereby yielding strong interfacial films (Lawal, 2004; Mundi and Aluko, 2012).

Nevertheless, as the standing time progressed, OPPPI's foam stability decreased to 62.5 ± 0.00% at the 90 min. This decrease suggests that the interfacial film was insufficiently cohesive and viscous to sustain stable air bubbles for an extended duration (Fidantsi and Doxastakis, 2001; Cai et al., 2020b). To achieve enduring foam stability, proteins must establish extensive interactions around air bubbles within the matrix (Jain and Anal, 2016).

In the context of commercial protein isolates, following a 10 min duration of standing at room temperature, both SPI (76.92 ± 1.187%) and PPI (64.10 ± 1.132%) exhibited decreased FS compared to OPPPI. Notably, over time, the foam had entirely disappeared within 10 min for PPI. Similarly, in the case of SPI, the foam progressively decreased until it vanished entirely after 30

min of standing.

Table XI: Foaming capacity of OPPPI, SPI, and PPI and their foaming stability over time.

	FC (%)	FS (%)			
		10 min	30 min	60 min	90 min
OPPPI	76.385 ± 1.251	87.8675 ± 0.519	68.3035 ± 0.631	63.3925 ± 1.262	62.5 ± 0.00
SPI	119.5 ± 2.121	82.36 ± 2.72	51.71 ± 1.94	/	/
PPI	58 ± 2.282	69.551 ± 2.72	/	/	/

Results are expressed as the mean of three experiments ± SD. FC: Foaming capacity, FS: Foaming stability, OPPPI: Optimized *Pinus pinea* L. protein isolate, PPI: Pea protein isolate, SPI: Soy protein isolate.

I.2.4. Water/oil holding capacities of OPPPI

WHC and OHC represent key protein attributes governing their ability to absorb water or oil. These characteristics render proteins suitable for deployment as additives in protein-containing foods, contributing to the enhancement of food quality. WHC and OHC can influence product shelf life, as well as texture and flavor, thus impacting overall product appeal and stability (Hou et al., 2017; Mohan and Mellem, 2020b; Zhang et al., 2020).

I.2.4.1. Water holding capacity

WHC of proteins primarily depends on the composition of amino acids within protein molecules (Aryee et al., 2018). This attribute is based on the interaction between water molecules and polar groups in polypeptide chains through hydrogen bonding (Liu et al., 2013), where highly charged proteins are more likely to exhibit stronger electrostatic affinity for water (Lam et al., 2018), thereby amplifying WHC (Li et al., 2018).

The WHC of OPPPI is presented in Table XII. OPPPI demonstrated a WHC of 1.151 ± 0.021 g H_2O/g, which was lower than values reported for Aleppo pine protein isolate (3.9 g/g) (Al-Ismail et al., 2018), sangri seed protein concentrate (3.87 g/g) (Garg et al., 2020), mung bean protein isolate (2.62 g/g) (Du et al., 2018), and hyacinth bean protein isolate (2.48 g/g) (Mohan and Mellem, 2020c). Nevertheless, it higher than the WHC of ginger bread plum seed protein isolate (0.94 g/g) (Amza et al., 2011). In comparison to commercial protein isolates, both soy protein isolate (3.0904 ± 0.016 g/g) and pea protein isolate (2.806 ± 0.120 g/g) exhibited higher WHC compared to OPPPI.

The poor WHC of OPPPI suggests a deficiency in hydrophilic amino acids within the protein structure, which serve as primary sites for water interaction (Sathe et al., 1982). This could also originates from a reduced density of surface charges, leading to the formation of a loose protein network with limited water entrapment (Cai et al., 2020b). Moreover, the extraction method employed might influence the WHC of proteins (Krause et al., 2002). Proteins obtained through alkali extraction and isoelectric precipitation may be subjected to a disruption during the process, exposing more hydrophobic sites and raising the risk of irreversible protein aggregation, consequently reducing water absorption capacity (Krause et al., 2002; Wu et al., 2009; Amza et al., 2011). For proteins to excel in retaining water, they must be capable of swelling, dissociating, and unfolding to reveal more binding sites (Khalid et al., 2003).

Table XII: Water and oil holding capacities of OPPPI, SPI, and PPI.

	WHC (g H_2O/g)	OHC (g oil/g)
OPPPI	1.151 ± 0.021	6.664 ± 0.0551
SPI	3.904 ± 0.016	0.144 ± 0.025
PPI	2.806 ± 0.120	0.253 ± 0.007

Results are expressed as the mean of three experiments ± SD. OHC: Oil holding capacity, WHC: water holding capacity, OPPPI: Optimized *Pinus pinea* L. protein isolate, PPI: Pea protein isolate, SPI: Soy protein isolate.

I.2.4.2. Oil holding capacity

OHC, as defined by Kinsella (1979), refers to the physical encapsulation of oil, measuring a protein's capability to bind fats onto the non-polar chains of proteins (Liu et al., 2013). Furthermore, OHC can mirror the protein's hydrophobic capacity (Du et al., 2018); proteins possessing high hydrophobic characteristics are prone to exhibit a strong propensity for retaining oil (Lam et al., 2018). The OHC is influenced by factors such as the matrix structure, macromolecule size, protein type, hydrophobicity, charge, and surface area (Wani et al., 2015; Lam et al., 2018; Mohan and Mellem, 2020b). High OHC in proteins contributes to the stabilization of lipid-rich foods by reducing the rate of oxidation (Li et al., 2018).

The OHC of OPPPI was measured at 6.664 ± 0.0551 g oil/g, which was comparable to the OHC observed for tree peony seed protein (6.93 g/g) (Gao et al., 2018) and was within the range reported for kidney bean isolates (5.5-7.2 g/g) (Shevkani et al., 2015). Conversely, lower OHC values were recorded for cashew nut isolate (4.42 g/g) (Ogunwolu et al., 2009), bell pepper protein

isolate (4.57 g/g) (Li et al., 2018), and Aleppo pine protein isolate (3.1 g/g) (Al-Ismail et al., 2018). Additionally, in comparison to commercial pea and soy protein isolates, OPPPI exhibited higher OHC. Specifically, soy and pea isolates demonstrated poor OHC of 0.144 ± 0.025 g/g and 0.253 ± 0.007 g/g, respectively.

The high OHC of OPPPI can be attributed to the presence of multiple non-polar side chains that facilitate oil retention through associative binding (Sathe et al., 1982). The abundance of hydrophobic amino acids is crucial for binding hydrocarbon chains of fats (Aryee et al., 2018). Moreover, proteins with high surface hydrophobicity tend to possess superior OHC (Cai et al., 2020b), as the exposure of hydrophobic amino acids on the surface enables effective oil entrapment (Mir et al., 2019a). Additionally, a higher protein content has been linked to enhanced OHC (Ogunwolu et al., 2009; Hou et al., 2017).

The high OHC of OPPPI and its comparatively lower WHC highlights the predominance of hydrophobic amino acids over hydrophilic amino acids, accentuating the lipophilic nature of OPPPI (Kaushik et al., 2016; Mohan and Mellem, 2020c).

I.2.5. Biochemical characterization of OPPPI

I.2.5.1. FTIR analysis of OPPPI

To gain a deeper understanding of the secondary structure and constituents of OPPPI, Fourier-transform infrared (FTIR) analysis was employed. This technique is widely recognized for assessing the secondary structure of proteins (Garidel and Schott, 2006; Akyuz et al., 2018).

A standard protein infrared spectrum typically exhibits nine distinctive amide bands (A, B, I-VII), featuring vibrational contributions from both the protein backbone and amino acid side chains (Barth, 2007; Kong and Yu, 2007). Among these, the most prominent vibrational bands associated with the protein backbone are amide I and amide II, originating from specific stretching and bending motions within the backbone. These bands are situated within the 1700-1600 cm^{-1} range (Amide I) and the 1480-1580 cm^{-1} range (Amide II) (Kong and Yu, 2007; Achouri et al., 2012; Dapčević-Hadnađev et al., 2019). The occurrence of amide I and amide II frequencies in the spectrum is influenced by the specific hydrogen bonding interactions between C=O and N-H groups. Different secondary structural arrangements (such as α-helix, β-sheet, β-turn, etc.) correspond to distinct hydrogen bonding patterns (Garidel and Schott, 2006; Kong and Yu, 2007).

The FTIR spectrum of OPPPI, spanning from 4000 to 400 cm^{-1}, is presented in Figure 17.

The FTIR spectrum of OPPPI is characterized by three distinct regions corresponding to the amide I, amide II, and amide III bands. A distinct peak emerged in the amide I region, centered at 1639 cm^{-1}, indicative of the intramolecular β-sheet structure. This peak arises primarily from the C=O stretching vibration of the amide group, weakly coupled with in-plane N-H bending and C-N stretching (Garidel and Schott, 2006). The amide I region is particularly sensitive to secondary structural components. In the amide II region, a less intense peak was observed at 1538 cm^{-1}, originating from in-plane N-H bending and C-N stretching modes. The amide II region is more complex compared to the amide I region, making it less effective for predicting protein structure. The amide III region, that occurs in 1450-1200 cm^{-1}, arises from a complex blend of vibrational modes involving in-phase N-H bending coupled with C-N stretching vibration, along with slight contributions from C-O in-plane bending and C-C stretching vibration (Barth, 2007). This region exhibited three faint peaks, one at 1243 cm^{-1}, associated with the β-sheet structure as suggested by Wei et al. (2018); another at 1400 cm^{-1}, attributed to symmetric C=O stretching of COO- (Böcker et al., 2017); and a third at 1450 cm^{-1}, resulting from the C-N stretching vibration.

The FTIR spectrum of OPPPI exhibited a broad peak centered at 3284 cm^{-1} (assigned to the amide A band), originating from N-H stretching vibration. Distinct and strong peaks corresponding to methyl (CH_3) and methylene (CH_2) groups in the aliphatic side chains of proteins were evident. The peak at 2968 cm^{-1} was attributed to the asymmetric stretching vibration of CH_3, while the peak at 2924 cm^{-1} was assigned to the asymmetric stretching vibration of CH_2. Additionally, a less intense peak was observed at 3667 cm^{-1}, associated with the O-H stretch of hydroxyl groups.

Regarding potential contaminants, a prominent peak at 1060 cm^{-1}, arising from C-O stretching, indicated the presence of carbohydrates. Meanwhile, a weaker peak at 1745 cm^{-1} was identified as the result of C=O stretching in esters.

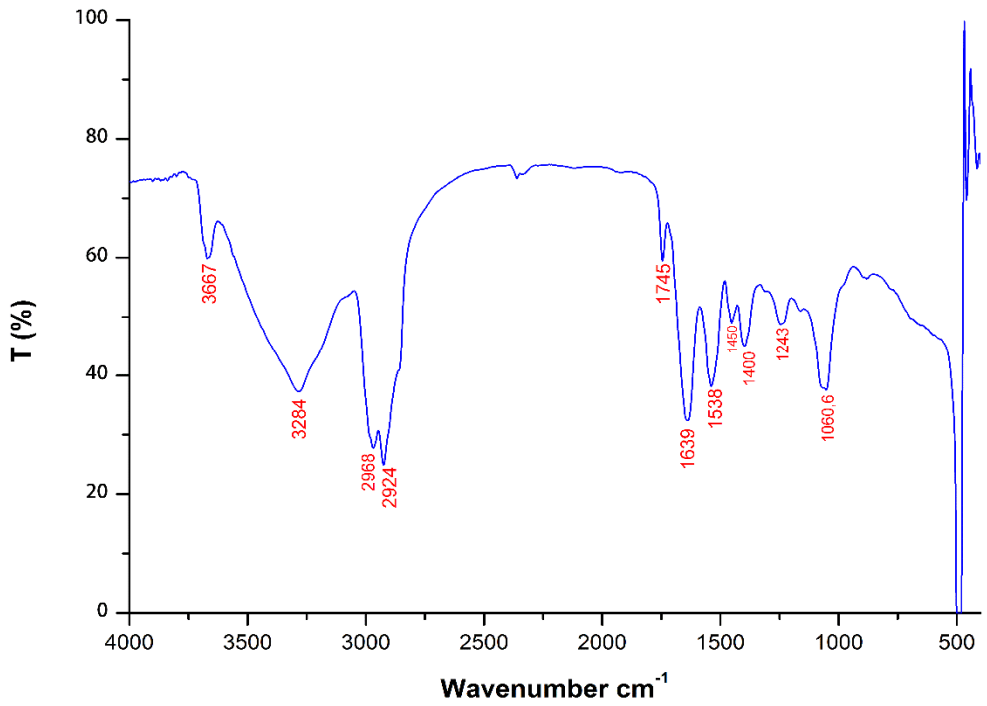

Figure 17: FTIR spectrum of optimized *Pinus pinea* L. pine nuts protein isolate.

The presence of aggregate peaks at 1618 cm^{-1}, 1683 cm^{-1}, and 1694 cm^{-1}, as previously found in hempseed proteins (Dapčević-Hadnađev et al., 2019), was not detected in the FTIR spectrum of OPPPI. This absence of aggregate peaks indicates that OPPPI exhibits reduced aggregation and maintains its structural integrity throughout the processing. Moreover, native proteins exhibit more intense peaks within the 1630-1660 cm^{-1} range [1] (Widjanarko et al., 2011). This was observed in the case of OPPPI, which displayed a distinct and significant peak at 1639 cm^{-1}.

I.2.5.2. SDS-PAGE electrophoresis of OPPPI

The SDS-PAGE analysis of OPPPI is shown in Figure 18, illustrating the protein profile under both reducing (lane 1) and non-reducing (lane 2) conditions. In the non-reduced sample, five distinct bands were identified, with molecular weights ranging from 64.02 KDa to 12.92 KDa. Upon comparison between the SDS-PAGE profiles of the reduced and non-reduced samples, certain protein bands emerged, and some disappeared, signifying the breakdown of high molecular weight proteins into lower MW subunits due to the influence of the reducing agent (2-ME). Notably, similarities were observed between the protein isolate's profile pattern and the globulin fraction of *Pinus koraiensis* nuts (Wu et al., 2014).

Prominent protein bands of around 48.62 kDa and 33.41 kDa were noticeable in both lanes, suggesting that these proteins lack disulfide bonds and are likely 7S globulins. This category of globulins is recognized for their absence of disulfide bonds, primarily due to the absence of cysteine residues. Consequently, they are incapable of forming disulfide bonds and instead rely on non-covalent interactions for structural stability (Meng and Ma, 2001; Orruno and Morgan, 2007; Barac et al., 2010).

A protein band with Mw of ~37.87 kDa could potentially correspond to the acidic subunit of the 11S globulin, while a protein band at 20.7 kDa may likely be attributed to the basic subunits of the 11S globulin. Studies have indicated that mature 11S globulins in peas consist of six subunit pairs that interact through non-covalent means. Each subunit pair comprises an acidic and basic subunit of approximately 40 kDa and 20 kDa, respectively, connected by a single disulfide bond (Barac et al., 2010). A broader band with an estimated weight of around 16.18 kDa was evident in lane 1, potentially representing the basic subunit of the 11S globulin. According to research by Chavan and Djurtoft (1982), the 11S globulin in black gram was found to decompose into five subunits ranging in molecular weight from 67 kDa to 16 kDa.

The protein band with an apparent weight of 64.02 kDa detected in the non-reduced sample is likely linked to the trimeric configuration of the 11S globulin (Barac et al., 2010). In the presence of reducing agents, this band tends to break down into three less pronounced bands. Conversely, when subjected to non-reducing conditions, these bands have a tendency to reassemble into the trimeric structure, appearing as a singular prominent band with the same molecular weight (Barac et al., 2010).

In lane 1 of the gel, two notable and prominent protein bands with MW ranging from 8.66-9.57 kDa and 10.06-10.75 kDa were evident; these subunits are likely associated with the 2S albumin. In lane 2, a substantial and intense band with a molecular weight of 12-15 kDa was similarly observed. It is evident that this protein band has co-fractioned, giving rise to the two prominent subunits detected in lane 1. Generally, 2S albumins consist of a pair of polypeptides (small and large subunits) connected by two disulfide bonds (Wang and Xiong, 2019). From a nutritional viewpoint, 2S albumins contain a notable amount of nitrogen and sulfur-containing amino acids, thereby offering a potential source of thiols for creating highly nutritious food formulations (de Souza Cândido et al., 2011; Wang and Xiong, 2019).

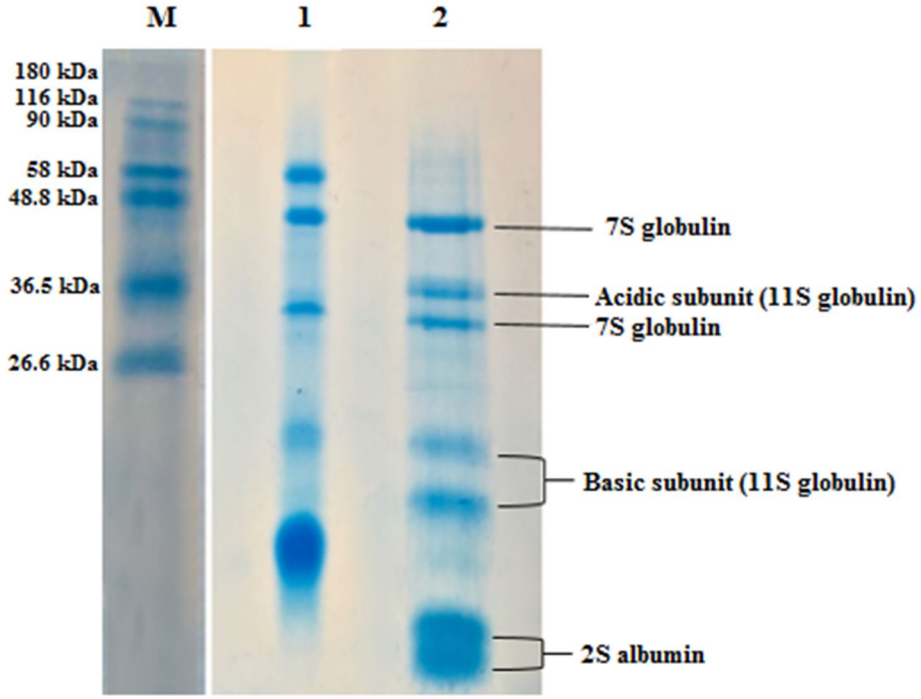

Figure 18: SDS-PAGE profile of OPPPI under non-reducing (1) and reducing conditions (2), M represents molecular weight marker.

Based on the SDS-PAGE profile observed for OPPPI, it can be concluded that the primary constituents of OPPPI are globulins and albumins. The absence of glutelin, which were indicated to constitute around 10% of the total storage proteins in pine nuts by Nasri and Triki (2007), could potentially be attributed to the pH of the extraction process. Srivastava and Roy (2011) proposed that both the extraction and precipitation pH could influence the protein composition. They further noted that proteins extracted at pH 8 were predominantly composed of globulins and albumins, while those obtained at pH 9 and higher exhibited the presence of glutelin and prolamins.

I.3. Conclusion

The present study aimed to optimize the alkali extraction process of *Pinus pinea* L. pine nuts protein isolates with the objective of enhancing their purity, solubility, and emulsifying activity. The results highlighted the considerable influence of extraction conditions, namely extraction pH, duration, and solvent-to-sample ratio, on the functional properties and purity of the protein isolates. The model-suggested and validated optimal conditions were an extraction pH of 8, a time of 108 min, and a solvent-to-sample ratio of 1:10 g/mL. A comparative assessment of functionalities was conducted between OPPPI and commercial SPI and PPI. In summary, OPPPI

demonstrated higher solubility, oil holding capacity, as well as foaming and emulsion stabilities when contrasted with commercial SPI and PPI. Emulsifying activity was comparable in all three cases. Additionally, OPPPI exhibited lower water holding capacity and foaming capacity in comparison to SPI, but higher foaming capacity than PPI. In view of these results, OPPPI holds potential as a promising food ingredient applicable to various food sectors, which can serve as a substitute for widely utilized plant-based alternatives within the food industry.

Chapter II. Antioxidant activity of Pinus pinea L. nuts protein hydrolysates produced using different enzymes

Chapter II. Antioxidant activity of *Pinus pinea* L. pine nuts protein hydrolysates produced using different enzymes

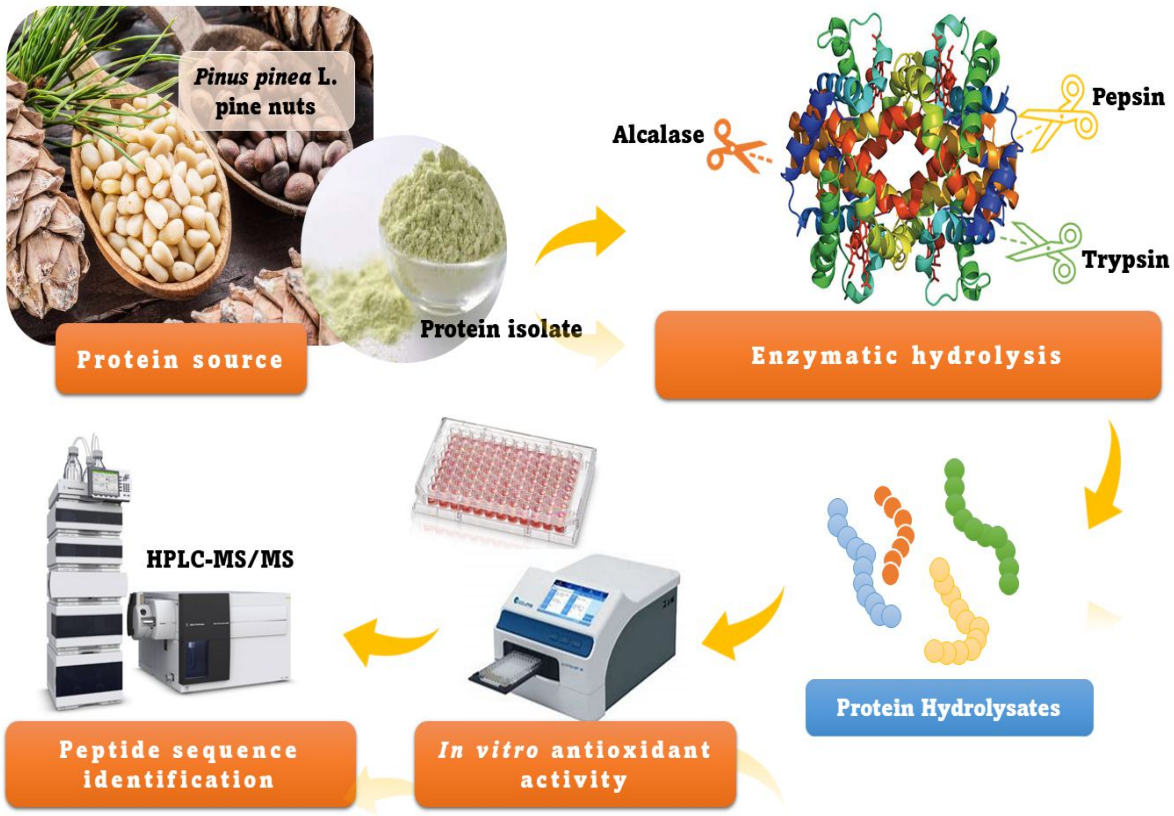

Figure 19: Graphical abstract representing the enzymatic hydrolysis of pine nuts protein isolate, the assessment of the antioxidant activity of the generated protein hydrolysates, and peptide sequence identification via HPLC-MS/MS.

Abstract

In recent times, bioactive peptides derived from food proteins have attracted considerable interest, as they are likely to have a positive impact on human health. *Pinus pinea* L. pine nuts have received particular interest for the production of bioactive peptides due to their well-documented beneficial effects on health upon consumption. In the present study, the production of pine nut hydrolysates was carried out using various enzymes, by both single enzymatic and sequential hydrolysis methods. Varied results were observed among the different hydrolysates generated, encompassing aspects such as degree of hydrolysis (DH), SDS-PAGE profiles, antioxidant activity and peptide sequences. Notably, the trypsin/Alcalase hydrolysate showed the highest DH, with a value of 47.70 ± 0.220%. All hydrolysates exhibited potent antioxidant activity. In particular, trypsin hydrolysate displayed remarkable performance in DPPH radical scavenging activity (IC_{50} = 68.75 ± 0.48 µg/mL), Fe^{2+} chelating activity (IC_{50} = 19.50 ± 0.008 µg/mL), and OH radical scavenging activity (IC_{50} = 21.32 ± 0.02 µg/mL). On the other hand, AH presented the highest ORAC value (1090.491 ± 4.136 µmol TE/g), PH demonstrated superior reducing power (OD_{700nm} = 1.227 ± 0.001 at 1069 µg/mL), and P/A H displayed the most effective ABTS radical scavenging activity (IC_{50} = 47.02 ± 0.20 µg/mL). SDS-PAGE analysis of the hydrolysates revealed that PH, AH, P/T H, P/A H, T/A H and P/T/A H showcased a single band. In contrast, TH showcased a distinct profile with six bands. By employing HPLC-MS/MS, five peptides (DQSQSYD, ALDQSQS, AEELPNR, DYLQR, REEEEAVERAE) were identified in the most bioactive hydrolysate (TH). These results strongly indicate that pine nuts protein hydrolysates hold potential as promising antioxidants, with implications for functional food applications and the prevention of oxidation reactions in food processing.

Keywords: *Pinus pinea* L. nuts, Protein hydrolysates, Antioxidant peptides, HPLC-MS/MS.

II.1. Materials and methods

II.1.1. Preparation of protein isolate (PI)

The process of preparing pine nut protein isolate was carried out following the procedure outlined in the preceding section (I.1.2.2.). This involved alkaline extraction and isoelectric precipitation under the optimal conditions: pH 8.0, an extraction duration of 108 min, and a substrate-solvent ratio of 1:10 g/mL.

II.1.2. Preparation of pine nuts protein hydrolysates by enzymatic hydrolysis

II.1.2.1. Principle of the method

Enzymatic hydrolysis is the most widely used technique for generating bioactive peptides from native proteins. Proteases, enzymes that break down protein's peptide bonds leading to the formation of smaller molecules, are the key players in this process. These proteases are categorized into two groups: endopeptidases, which cleave peptide bonds internally, resulting in the release of peptides with varying molecular weights, and exopeptidases, which cleave peptide bonds at the protein's outer ends, yielding individual amino acids. Exopeptidases can further be divided into aminopeptidases, which break bonds from the protein's N-terminal end, and carboxylpeptidases, which cleave bonds from the C-terminal end (Figure 20) (Saadi et al., 2015).

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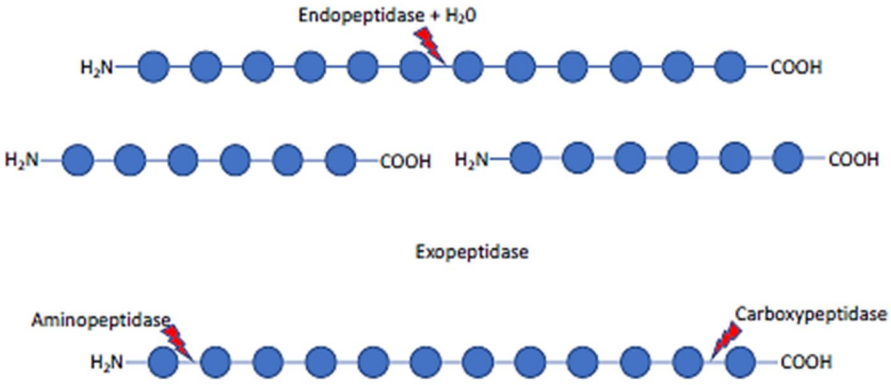


Figure 20: Different enzyme families based on their mode of action.

Food-grade proteases can be derived from different sources including microorganisms (e.g., Alcalase, Flavorzyme, and Protamex), plants (Papain), and animals (pepsin and trypsin).

Endopeptidases are subdivided into four families, based on their mechanism of action and the specific presence of amino acids or a metal atom in the active site:

- **Serine proteases:** These enzymes feature a catalytic triad consisting of three amino acids (Ser-His-Asp), examples being trypsin and chymotrypsin.
- **Cysteine proteases:** This category is characterized by the presence of cysteine in their active site (e.g., Papain).
- **Acid proteases:** These proteases possess aspartic acid within their active site (e.g., pepsin).
- **Metalloproteases:** This group contains a metal cation, typically zinc, in their structure, which activates the water molecule required for protein hydrolysis (e.g., Thermolysin).

II.1.2.2. Preparation of the protein hydrolysates

In the current study, pine nuts protein hydrolysates were produced utilizing three distinct enzymes (pepsin, trypsin, and Alcalase), each with their respective characteristics as outlined in Table XIII. Enzymatic hydrolysis of pine nuts protein isolates was achieved by single enzyme hydrolysis, using pepsin, trypsin, and Alcalase solely, and by sequential hydrolysis, using different combinations of the aforementioned enzymes (pepsin/trypsin (P/T), pepsin/Alcalase (P/A), trypsin/Alcalase (T/A), pepsin/trypsin/Alcalase (P/T/A)).

Table XIII: Characteristics of the enzymes used in this study.

Enzyme	Origin	Family	Optimal pH	Optimal T (°C)
Pepsin	Animal (porcine)	Endopeptidase (Aspartic acid protease)	2	37
Trypsin	Animal (bovine)	Endopeptidase (Serine protease)	8	37
Alcalase	Microorganism (*Bacillus licheniformis*)	Endopeptidase (Serine protease)	8	55

Enzyme hydrolysis took place in a shaking incubator. Before starting the hydrolysis process, protein isolate suspensions were prepared in suitable buffers specific to the chosen enzyme. These suspensions were then preheated to the optimum temperature for 10 minutes, after which the pH was adjusted. The enzymes were then introduced to initiate the hydrolysis process.

II.1.2.2.1. Single enzyme hydrolysis

For pepsin hydrolysis, a suspension of PI was prepared in KCl/HCl buffer at a

concentration of 5 mg/mL and adjusted to pH 2. Pepsin from porcine gastric mucosa was then added at an enzyme-substrate ratio (E/S) of 128.034 U/mg protein. This mixture underwent hydrolysis for 4 hours at 37°C. Similarly, trypsin hydrolysis involved preparing the PI suspension in 50 mM phosphate buffer, pH 8 at a concentration of 5 mg/mL. Following pH adjustment, trypsin from bovine pancreas was added at an E/S ratio of 0.377 U/mg protein, and the hydrolysis proceeded for 4 hours at 37°C. For Alcalase hydrolysis, the PI suspension was prepared at 30 mg/mL in 50 mM phosphate buffer, pH 8, with Alcalase® 2.4 L added at an E/S ratio of 62.205 U/mg protein after pH adjustment. The hydrolysis was conducted for 3 hours at 55°C (Zhao et al., 2011)**.**

II.1.2.2.2. Sequential hydrolysis

The same hydrolysis conditions mentioned above (pH, T, substrate concentration) were adopted for all enzyme combinations (P/T, P/A, T/A, P/T/A). The initial enzyme was introduced into the PI suspension and allowed to hydrolyze for 2 hours. Subsequently, pH and temperature were adjusted so that the next enzyme could be added and hydrolyze for an additional 2h.

For all samples (prepared by single or sequential hydrolysis), once the hydrolysis was complete, the reaction mixtures were heated to 85°C for 15 minutes to deactivate the enzymes. After cooling, the hydrolysates were centrifuged at 8,000 × g for 15 minutes at 4°C. Supernatants were collected and lyophilized for subsequent analysis (Polanco-Lugo et al., 2014).

II.1.3. Determination of total protein content

The total nitrogen content of PI and protein hydrolysates was measured using LECO chns-932 elemental analyzer, this analyzer is able to determine the amount of nitrogen, carbon, hydrogen, and sulfur. To calculate total protein content, a conversion factor of 6.25 was used.

II.1.4. Determination of the degree of hydrolysis (DH)

The degree of hydrolysis refers to the percentage of peptide bonds that have been cleaved. The trinitrobenzenesulfonic acid (TNBS) method is used to determine the concentration of primary amino groups in the hydrolysate. Essentially, this technique utilizes a spectrophotometric assay to measure the formation of the chromophore resulting from the reaction between TNBS and primary amines (Figure 21). The absorbance of this product is measured at 340 nm. The reaction occurs under slightly alkaline conditions and is stopped by reducing the pH. It is important to note that TNBS can also react slowly with hydroxyl ions, causing an increase in the blank reading, and this increase can be influenced by light (Adler-Nissen, 1979).

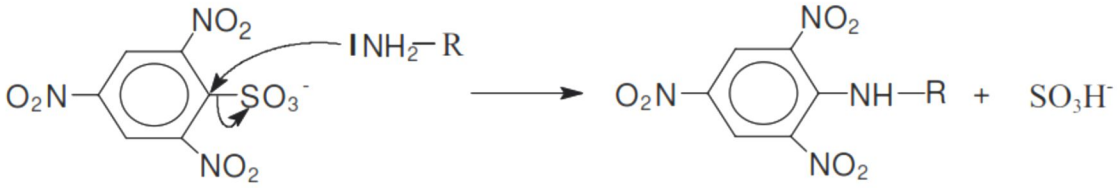


Figure 21: Reaction of TNBS with amino groups Adler-Nissen (1979).

The degree of hydrolysis of protein hydrolysates was determined using the TNBS method, as described by Adler-Nissen (1979). To carry out this analysis, all hydrolysate samples and L-leucine standard solutions (with concentrations ranging from 0.1-2 mM) were prepared in 1% (w/v) SDS. Subsequently, 125 µL aliquots from each sample, diluted to a proper concentration, were added to 1 mL of 0.2125 M phosphate buffer, pH 8.20. Afterwards, a volume of 1 mL of TNBS solution (0.1% (v/v) in water) was then added to the tubes, which were then shaken and incubated for 1h at 50°C in a covered water bath to prevent exposure to light. After incubation, 2 mL of 0.1 N HCl were added to each tube to halt the reaction. After the samples cooled at room temperature for 30 min, the absorbance was measured at 340 nm a UV-Visible spectrophotometer. The DH was calculated using the following formula:

$$DH\ (\%) = 100\ (h/h_{tot})$$

Where, h represents the concentration of α-amino groups formed during hydrolysis process (L-leucine mEq/g protein), and h_{tot} represents the total amount of amino groups present in the protein isolate (mEq/g protein).

The total amount of amino groups in the protein isolate was determined by carrying out a total hydrolysis of the sample in 6 N HCl at 110°C for 24h.

II.1.5. SDS-PAGE analysis of the protein hydrolysates

The SDS-PAGE analysis of the protein hydrolysate samples and PI (used as a control) was carried out following the Laemmli (1970) method, with some modifications. Briefly, the samples were dissolved in a sample buffer (tris-HCl 0.05 M pH 6.8, SDS 1.6% w/v, glycerol 8% v/v, bromophenol blue 0.002% w/v, and β-mercaptoethanol 2% v/v) and subsequently heated at 95°C for 5 min. After cooling down, 30 µL (equivalent to 30 µg protein/well) of each sample were loaded onto 4-12% Bis-Tris Criterion XT precast gel using 1% (v/v) XTMES running buffer (BIO-Rad Laboratories, Richmond, CA, USA). The samples were run at 100 V for 15 min, then at 150

V for 30 min. The gel was stained for 1h using Bio-safe Coomassie blue R-250 (BIO-Rad Laboratories, Richmond, CA, USA) and subsequently destained using MilliQ water. Precision plus Protein TM Dual Xtra Prestained Protein Standards (BIO-Rad Laboratories, Richmond, CA, USA) was used as a molecular weight marker.

II.1.6. Evaluation of antioxidant activity

The antioxidant activity of pine nut protein hydrolysates was evaluated using six distinctive assays, including DPPH radical scavenging activity, ABTS radical scavenging activity, OH radical scavenging activity, Oxygen radical absorbance capacity assay, Ferreous iron chelating activity, and Ferric reducing power.

II.1.6.1. DPPH radical scavenging activity

The assessment of the ability of protein hydrolysates to scavenge DPPH radicals was determined based on the method described by Farvin et al. (2014), with some modifications. Protein hydrolysates samples were prepared in a 50 mM phosphate buffer, pH 7.4 at different concentrations. In Eppendorf tubes, 176 µL aliquots of each sample were mixed with 1000 µL of DPPH solution (0.1 mM in methanol). The mixtures were incubated for 30 min in the dark at room temperature, then centrifuged at 3000 × g for 5 min (as protein precipitation will occur). Afterward, aliquots of 200 µL of each supernatant was transferred to a 96-well microplate. The absorbance was recorded at 515 nm in a BioTek cytation 5 imaging reader. The percentage of DPPH radical scavenging was calculated using the following formula:

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

For the control sample, methanol was added in place of the samples. For each sample, the IC_{50} value, representing the concentration required to scavenge 50% of the free radical, was determined.

II.1.6.2. $ABTS^{\bullet+}$ radical scavenging activity

The $ABTS^{\bullet+}$ radical scavenging activity of protein hydrolysates was assessed according to the method described by Zhang et al. (2021), with slight modifications. The ABTS radical ($ABTS^{\bullet+}$) solution was prepared by mixing equal volumes (v/v) of 7 mM of ABTS and 2.45 mM of potassium persulfate. The mixture was left to react for 12-16 h in the dark at room temperature before use. The resultant $ABTS^{\bullet+}$ stock solution was diluted in 50 mM phosphate buffer, pH 7.4

until the absorbance at 734 nm reached 0.700. Hydrolysate samples were prepared in phosphate buffer (50 mM, pH 7.4) at various concentrations. In a 96-well polystyrene microplate, a 10 µL volume of the samples was mixed with 200 µL of freshly diluted ABTS•+ solution. The mixtures were incubated for 1h in the dark at room temperature, and absorbance was measured at 734 nm using a BioTek cytation 5 imaging reader.

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

The control consists of a mixture of 10 µl PB (50 mM, pH 7.4) and 200 µl $ABTS^{\bullet+}$ solution. The IC_{50} value was determined for each sample.

II.1.6.3. Hydroxyl radical scavenging activity

The OH radical scavenging activity of protein hydrolysates was determined according to the method described by Mintah et al. (2019), with slight modifications. Briefly, 50 µL of samples, prepared at different concentrations, were mixed with 50 µL of 6 mM iron (II) sulfate ($FeSO_4$) solution and 50 µL of 6 mM H_2O_2 solution. The mixtures were left to incubate at room temperature for 15 min, and then 50 µL of 6 mM sulfosalicylic acid dihydrate solution were added. After 30 min of incubation in the dark at room temperature, the absorbance was measured at 510 nm. The OH radical scavenging activity (%) was calculated using the following equation:

$$(\%)\ \text{inhibition} = [(1-(A_{sample}-A_{control})/A_{blank}] \times 100$$

The control sample was devoid of sulfosalicylic acid dihydrate, while samples were replaced by MilliQ water in the case of the blank.

II.1.6.4. Oxygen radical absorbance capacity (ORAC) assay

The ORAC of protein hydrolysates was evaluated following to the method described by Gillespie et al. (2007), with slight modifications. The assay was performed in a 96-well black polystyrene microplate with a clear bottom. Protein hydrolysate samples were prepared in 75 mM phosphate buffer, pH 7.4, at concentrations of 37.5 µg/mL, 75 µg/mL, and 150 µg/mL. As a first step, 150 µL of 0.08 µM fluorescein solution were loaded into each well, followed by the addition of 25 µL of sample, or 75 mM of phosphate buffer (in the case of the blank), or Trolox, which was used as the antioxidant standard (6.25 µM, 12.5 µM, 25 µM and 50 µM). The plate was pre-incubated for 10 min at 37°C, then to initiate the reaction, 25 µL of freshly prepared 150 mM AAPH solution was added to each well. The kinetic read of the fluorescence was recorded

immediately at an excitation wavelength of 485 nm and an emission wavelength of 530 nm for a total of 90 min at 37°C using a BioTek cytation 5 imaging reader (BioTek instrumenets, USA).

The area under the decay (AUC) curve was calculated as follows:

$$AUC = 1 + f_1/f_0 + f_2/f_0 + f_3/f_0 + \ldots\ldots + f_{90}/f_0$$

Where f_0 represents the initial fluorescence recorded at time 0 min, and f_i represents the fluorescence recorded at time i min. The net AUC was determined by subtracting the AUC of the blank from that of the samples. ORAC values were expressed as µM Trolox equivalents per gram protein (µM TE/g protein).

II.1.6.5. Ferrous ion (Fe^{2+}) chelating activity

The Fe^{2+} chelation activity of protein hydrolysates was evaluated following the method described by Xia et al. (2012). In concise, 200 µL aliquots of samples prepared at different concentrations in MilliQ water were added in a 96-well polystyrene microplate, and then 6.66 µL of 2 mM iron (II) chloride ($FeCl_2$) were added. The mixture was incubated for 5 min at room temperature, followed by the addition of 13.3 µL of 5 mM Ferrozine solution. The microplate was shaken in a plate shaker and the mixture was incubated for a second time for 10 min in the dark at room temperature. The absorbance was recorded at 562 nm. The chelation activity percentage was measured as follows:

$$(\%)\ \text{inhibition} = [\ (1-(A_{sample}-A_{control})/A_{blank}] \times 100$$

Control and blank samples were prepared following the same procedures as the protein hydrolysate samples, except that the control sample was devoid of Ferrozine, and in the blank sample, the protein hydrolysate was substituted with MilliQ water. The IC_{50} value was determined for each sample and expressed on µg/mL.

II.1.6.6. Ferric reducing antioxidant power (FRAP)

The FRAP of the hydrolysates was assessed following the method outlined by He et al. (2013), with slight modifications. In concise, 250 µL of samples (prepared at different concentrations) were added to Eppendorf tubes and mixed with 250 µL of phosphate buffer (0.2 mM, pH 6.6) and 250 µL of 30.37 mM Potassium hexacyanoforate. The mixture was incubated at 50°C for 20 min. Afterward, to stop the reaction, 250 µL of 612.03 mM TCA were added, and to remove the precipitate formed, the resulting mixture was centrifuged (3000 × g, 10 min).

Subsequently, a 125 µL volume of supernatants were transferred to a 96-well polystyrene microplate, and 25 µL of 12.33 mM iron (III) chloride ($FeCl_3$) solution was added along with 100 µL of MilliQ water. The absorbance was measured at 700 nm.

II.1.7. HPLC coupled to tandem mass spectrometry (HPLC-MS/MS)

HPLC coupled to tandem mass spectrometry (HPLC-MS/MS) was carried out in Agilent 1100 HPLC system (Agilent Technologies, Waldbron, Germany), equipped with a Mediterranean Sea C18 column (150 × 2.1 mm) (Teknokroma, Barcelona, Spain), and connected to an Esquire 3000 linear iron trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization source. Freeze-dried Samples (Trypsin hydrolysate, pepsin/trypsin hydrolysate, and trypsin/Alcalase hydrolysate) were reconstituted in solvent A (water: formic acid, 100:0.1 v/v) and centrifuged, if necessary, 50 µL of supernatant was injected for each sample. A linear gradient from 0 to 45% of solvent B (Acetonitrile: formic acid, 100:0.1 v/v) for 120 min was performed for peptide elution and the flow was set at 0.2 mL/min. Spectra was recorded over the mass/charge (m/z) range of 100-1200 and the target mass was set at 900 m/z.

Data processing was done by using Data Analysis (version 4.0, Bruker Daltonics GmbH, Bremen, Germany). Peptide identifications were obtained using the MASCOT protein search engine (Matrix Science, London, UK; version 2.4) against Pin p1 sequence, the major allergen detected in pine nuts (UniProt database).

II.1.8. Statistical analysis

All experiments were performed in triplicate (n=3), and one-way analysis of variance (ANOVA) tests (GraphPad Prism software version 8.0.2) were used to compare mean values. Significant differences between the means were evaluated using Tukey's multiple comparisons test ($p < 0.05$).

II.2. Results and discussion

II.2.1. Total protein content and degree of hydrolysis

The total protein content (%) was calculated based on the nitrogen content (%) of samples, using a conversion factor of 6.25. The nitrogen content (%) and total protein content (%) of PI and protein hydrolysates are presented on Table XIV and Figure 22, respectively.

The protein content varied significantly ($p < 0.001$) from one protein hydrolysate to another, and a decrease in the protein content of the protein hydrolysates was observed compared with the PI, which had a protein content of 85.895%. Among protein hydrolysates, trypsin

hydrolysate had the lowest protein content at 26.343 ± 0.025%, while Alcalase hydrolysate had the highest protein content (58.625 ± 0.096%).

Table XIV: Nitrogen content of PI and protein hydrolysates.

Sample	PI	PH	TH	AH	P/T H	P/A H	T/A H	P/T/A H
N (%)	13.743 ± 0.060	6.84 ± 0.01	4.215 ± 0.025	9.38 ± 0.096	6.3 ± 0.03	7.405 ± 0.045	9.255 ± 0.085	8.01 ± 0.00

This variability in protein content of the hydrolysates could be attributed to the difference in the protein content of the initial material (substrate) and potential increases in the ash content due to pH adjustment using NaOH or HCl. Thiansilakul et al. (2007) reported a high ash content for round scad protein hydrolysates (24.56%), associating it with the use of McIlvaine buffer required for pH adjustment and control during enzymatic hydrolysis.

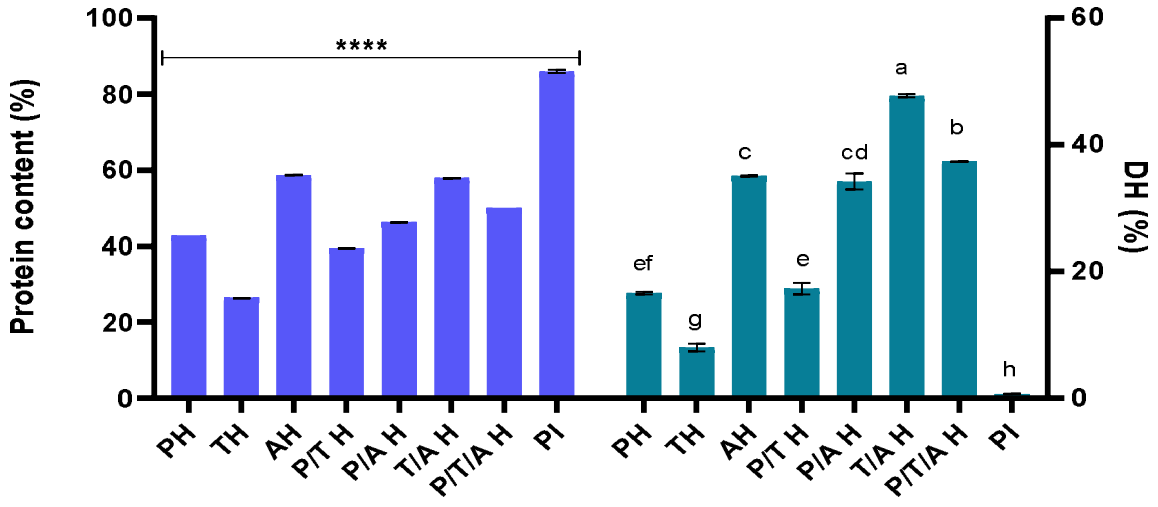


Figure 22: Protein content (%) and degree of hydrolysis (%) of the protein hydrolysates. Different letters (a–f) show significant differences ($p < 0.05$) between hydrolysates.

Seven distinct protein hydrolysates were produced using endopeptidases - pepsin, trypsin, and Alcalase. These enzymes were applied either individually in a single hydrolysis process or sequentially. The degree of hydrolysis (%) of pine nut protein hydrolysates is shown in Figure 22. Significant variations (< 0.0001) in DH were observed among the protein hydrolysates. The highest DH (47.70 ± 0.220%) was attained through sequential hydrolysis using trypsin and

Alcalase (T/A), followed by the P/T/A hydrolysate (37.34 ± 0.069%). In the case of single hydrolysis, the highest DH was achieved from Alcalase (35.07 ± 0.088%), whereas trypsin hydrolysate exhibited the lowest DH (8.0039 ± 0.639%) across all hydrolysates.

The results imply distinct affinities of the chosen enzymes for proteolytic cleavage. The elevated DH in T/A, P/A, and P/T/A hydrolysates, obtained through sequential hydrolysis, could be attributed to the initial presence of pepsin and trypsin, which generated pre-digested proteins, thereby creating more N-terminal sites susceptible to Alcalase's activity - the final enzyme introduced (Villanueva et al., 1999). Additionally, among the sequentially obtained hydrolysates, P/T hydrolysate displayed the lowest DH (17.28 ± 0.875%). Variations in DH values might stem from variations in the enzyme-to-substrate (E/S) ratio (Batish et al., 2020). Alcalase and trypsin were utilized at E/S ratios of 62.205 U/mg and 0.377 U/mg, respectively. Conversely, despite using a higher E/S ratio of 128.034 U/mg for pepsin, its hydrolysate exhibited lower DH, possibly due to the slower hydrolysis process of pepsin, necessitating extended timeframes to achieve higher DH levels.

II.2.2. SDS-PAGE analysis

The SDS-PAGE analysis of protein hydrolysates, DF, and PI is presented in Figure 23. The SDS-PAGE profiles of hydrolysates correspond to the extent of their hydrolysis. For instance, TH, characterized by the lowest DH, exhibits an SDS-PAGE profile consistent with its limited hydrolysis. In line with this, TH, being the least hydrolyzed, showcased six distinctive bands, among which some are prominently defined while others appear more subtly. Notably, this includes two pronounced bands with molecular weights of ~20 kDa and ~21 kDa, as well as two other clearly discernible bands at approximately ~5-9 kDa and ~11 kDa. Additionally, two faint bands are evident around ~14 kDa and ~16 kDa. On the other hand, PH, exhibiting a DH of 16.61%, presents a solitary substantial band with a molecular weight of approximately ~6-15 kDa.

For the other protein hydrolysates, the bands appearing in PI and DF (from 20 kDa to 50 kDa) were completely hydrolyzed and a single band appeared for AH (7-11 kDa), P/T H (9-11 kDa), P/A H (6-13 kDa), T/A H (9-10 kDa), and P/T/A H (9-12 kDa). The 7S and 11S globulins (from 20 kDa to 50 kDa) that were initially observed in PI and DF underwent complete hydrolysis as a result of enzymatic action. In contrast, the 2S albumin band (6-11 kDa) resisted enzymatic cleavage, although its prominence was not as pronounced as in PI and DF, it is possible that this band was partially hydrolyzed, leading to the loss of certain peptides. The 2S albumin band was

identified by Cabanillas et al. (2016) as Pin p1, the main allergen in *Pinus pinea* L. nuts; it was also reported to be thermoresistant and resistant to cleavage by digestive enzymes.

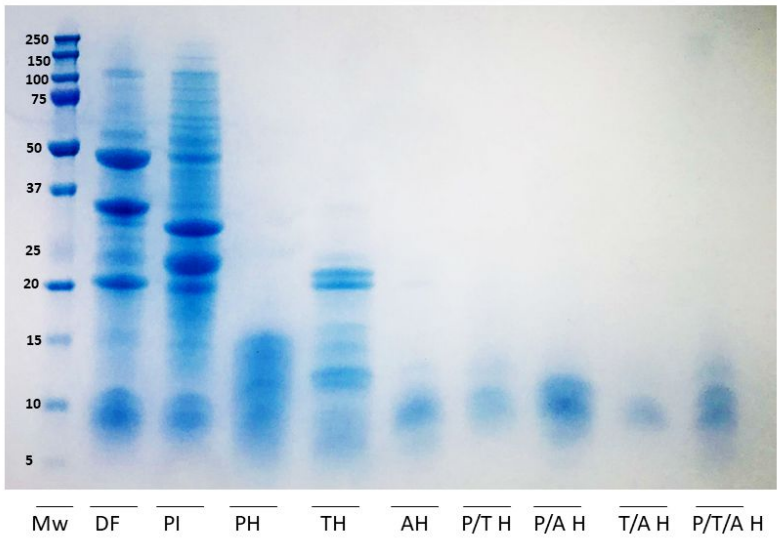


Figure 23: SDS-PAGE profile of pine nuts protein hydrolysates (PH, TH, AH, P/T H, T/A H, and P/T/A H), defatted flour (DF), and protein isolate (PI).

II.2.3. Antioxidant activities

To gain insight into the antioxidant potential of hydrolysates, various assays were conducted. Figure 24 illustrated the different antioxidant activities of hydrolysates tested at different concentrations. All results indicated that the antioxidant activities were dose dependent, and it displayed variability among different hydrolysates.

II.2.3.1. DPPH radical scavenging activity

DPPH compound, known for its relative stability, has been extensively employed to assess the capability of substances to function as scavengers of free radicals or donors of hydrogen ions. This approach is used to evaluate antioxidant activity. As depicted in Figure 24.a, the DPPH radical scavenging activity of hydrolysates demonstrated significant variation ($p < 0.0001$) depending on the protein hydrolysate. Notably, trypsin hydrolysate exhibited the highest activity. Upon comparing all protein hydrolysates, it becomes evident that those with the lowest degree of hydrolysis exhibited the most effective DPPH scavenging activity. For instance, TH and P/T H, both with the lowest degree of hydrolysis, showed superior activity, while hydrolysates resulting from sequential hydrolysis, and which had higher degrees of hydrolysis, displayed lower DPPH

scavenging activity. This observation aligns with the notion that a high degree of hydrolysis could diminish antioxidant activity**.** Overall, all hydrolysates demonstrated good DPPH scavenging activity, with IC_{50} values ranging from 68.75 ± 0.48 µg/mL (recorded for TH) to 210.75 µg/mL (recorded for P/A H) (Figure 25.a).

Recent studies on antioxidant peptides have shown that their activity mainly depends on bioactive amino acids that function as electron or hydrogen donors (Sila and Bougatef, 2016), with sequence position and hydrophobicity also playing a role (Wattanasiritham et al., 2016). Hydrophobic (e.g., Val, Leu) and aromatic amino acids (Phe, His, Tyr and Trp) can increase the radical-scavenging capabilities of peptides (Aluko and Monu, 2003). The hydrophobic nature of peptides facilitates interactions with hydrophobic targets, cellular membrane penetration, and solubility in lipids. This enhances contact with hydrophobic radical species. Numerous studies have indicated that the sequences of peptides and the positions of amino acids within those sequences are pivotal for their antioxidant characteristics (Zhang et al., 2014; Guo et al., 2015). Particularly, the hydrophobic attributes of amino acids such as Ala, Leu, Met, and Phe at the C- and N-termini contribute to peptide antioxidant activity (Samaranayaka and Li-Chan, 2011). Power et al. (2013) reported that hydrophobic amino acid Phe possessed hydrogen abundance in its methyl group, specifically an allylic hydrogen prone to facile abstraction by free radicals.

II.2.3.2. $ABTS^{\bullet+}$ radical scavenging activity

The water-soluble free radical $ABTS^{\bullet+}$ can easily react with antioxidants in aqueous environments through a proton transfer mechanism (Chen et al., 2012). Among all hydrolysates, increasing protein concentration led to greater $ABTS^{\bullet+}$ radical scavenging. Overall, the hydrolysates displayed substantial ABTS scavenging ability. The $ABTS^{\bullet+}$ scavenging activity of the seven hydrolysates ranked as follows: P/A>P/T/A>T/A>AH>PH>TH>P/T. It has been reported that amino acid sequence, peptide composition, and structural properties play a vital role in the antioxidant activity (Sampath Kumar et al., 2012). The effective antioxidant activity might arise from the presence of hydrophobic and aromatic amino acids in different proportions within the peptide sequence, which have been shown to enhance radical scavenging (Zhang et al., 2019). The concentration (IC_{50}) needed to neutralize 50 % of the $ABTS^{\bullet+}$ radical was calculated for each hydrolysate and is illustrated in Figure 25.b. The IC_{50} values significantly varied among

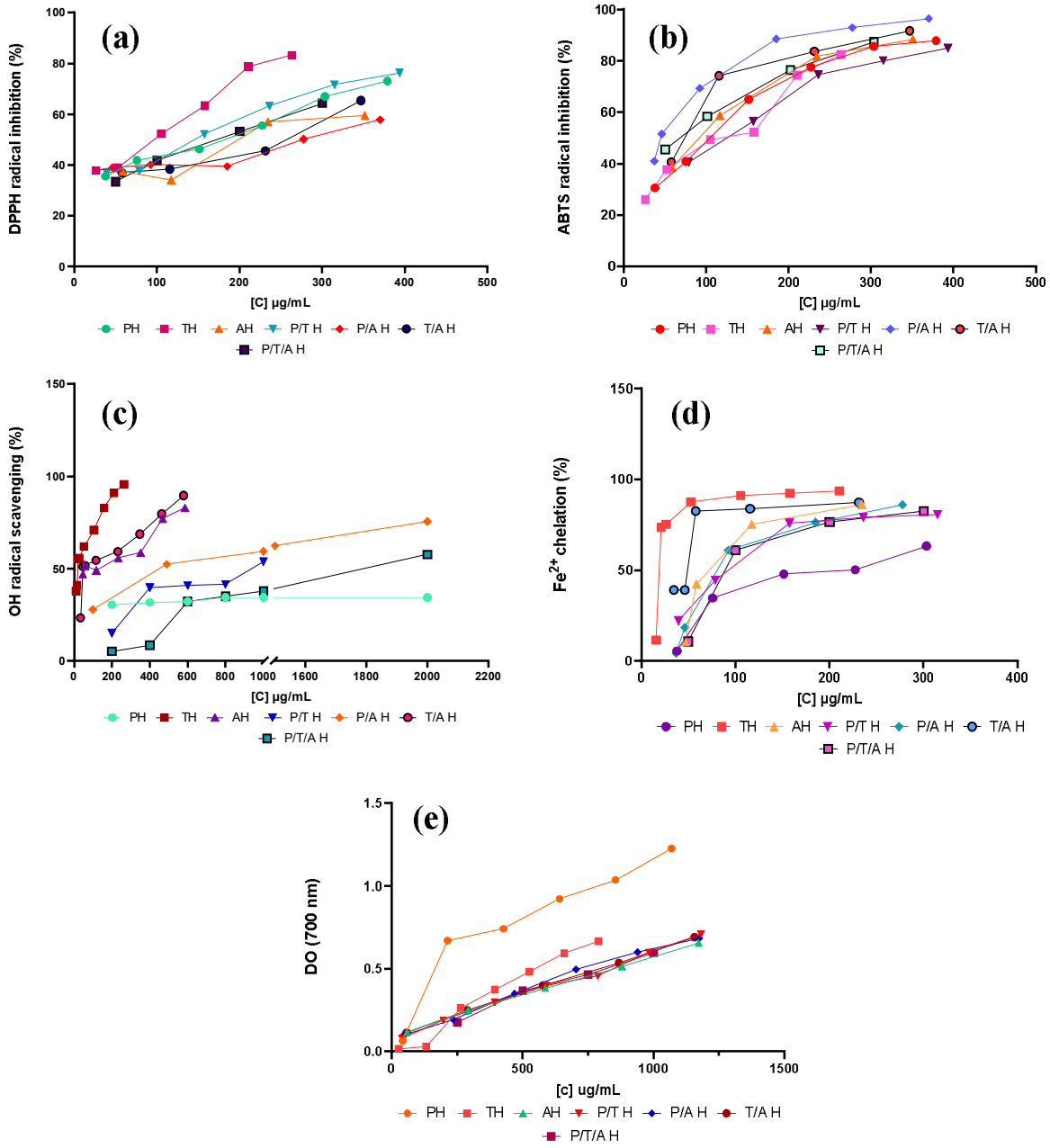


Figure 24: Antioxidant activity of the different hydrolysates measured by DPPH radical scavenging activity (a), $ABTS^{\bullet+}$ radical scavenging activity (b), OH radical scavenging activity (c), Fe^{2+} chelating activity (d), and reducing power assay measured at 700 nm (e).

hydrolysates ($p < 0.0001$). The highest ABTS activity was observed in the P/A H hydrolysate (IC_{50} = 47.02 ± 0.20 µg/mL), whereas the lowest $ABTS^{\bullet+}$ scavenging activity was recorded in the P/T hydrolysate (IC_{50} = 112.20 ± 0.1 µg/mL). It is worth noting that this result is

considerably higher than the reported IC_{50} for Chinese chestnut hydrolysate (15.25 mg/mL) and its fractions (8.65-17.94 mg/mL) (Feng et al., 2018), as well as the fraction obtained through ultrafiltration of pecan protein hydrolysate (74.35 % at 1 mg/mL) (Hu et al., 2018).

II.2.3.3. Hydroxyl radical scavenging activity

This assay evaluates the capacity of a substance to counteract the hydroxyl radical (·OH), a reactive oxygen species that readily reacts with biomolecules like amino acids, proteins, and DNA (Cacciuttolo et al., 1993). Consequently, eliminating the hydroxyl radical is likely a highly effective defense mechanism for a living organism against various diseases (Qian et al., 2008). The OH radical scavenging activity of the hydrolysates varied significantly ($p < 0.0001$) from one hydrolysate to another.

TH, AH, and T/A H displayed the highest OH radical scavenging activity, with IC_{50} values of 21.32 ± 0.02 µg/mL, 77.66 ± 0.04 µg/mL, and 80.45 ± 0.075 µg/mL, respectively. This suggests that these hydrolysates are effective antioxidants for scavenging OH radicals. In contrast, P/A H displayed a lower OH radical scavenging activity (IC_{50} = 479.35 ± 16.65 µg/mL), while P/T H and P/T/A H had a much lower OH radical scavenging activity (IC_{50} = 931.75 ± 7.25 µg/ml and 1736.5 ± 7.25 µg/mL, respectively). On the other hand, PH exhibited no OH radical scavenging activity, with the OH radical scavenging percentage remaining constant even with increasing the concentration to 2000 µg/mL. This indicates that pepsin hydrolysate lacks potential as OH radical scavenger. Hydrogen peroxide (H_2O_2), often used as a source of oxygen-derived free radicals in experimental, can generate highly reactive hydroxyl radicals (·OH) through a Fenton reaction when it interacts with transition metal ions like iron or copper. These hydroxyl radicals are extremely reactive and can cause significant damage to biomolecules, including proteins, lipids, and DNA. In the body, excessive production of hydrogen peroxide and the subsequent formation of hydroxyl radicals can contribute to oxidative stress and lead to various harmful effects (Wu et al., 1996).

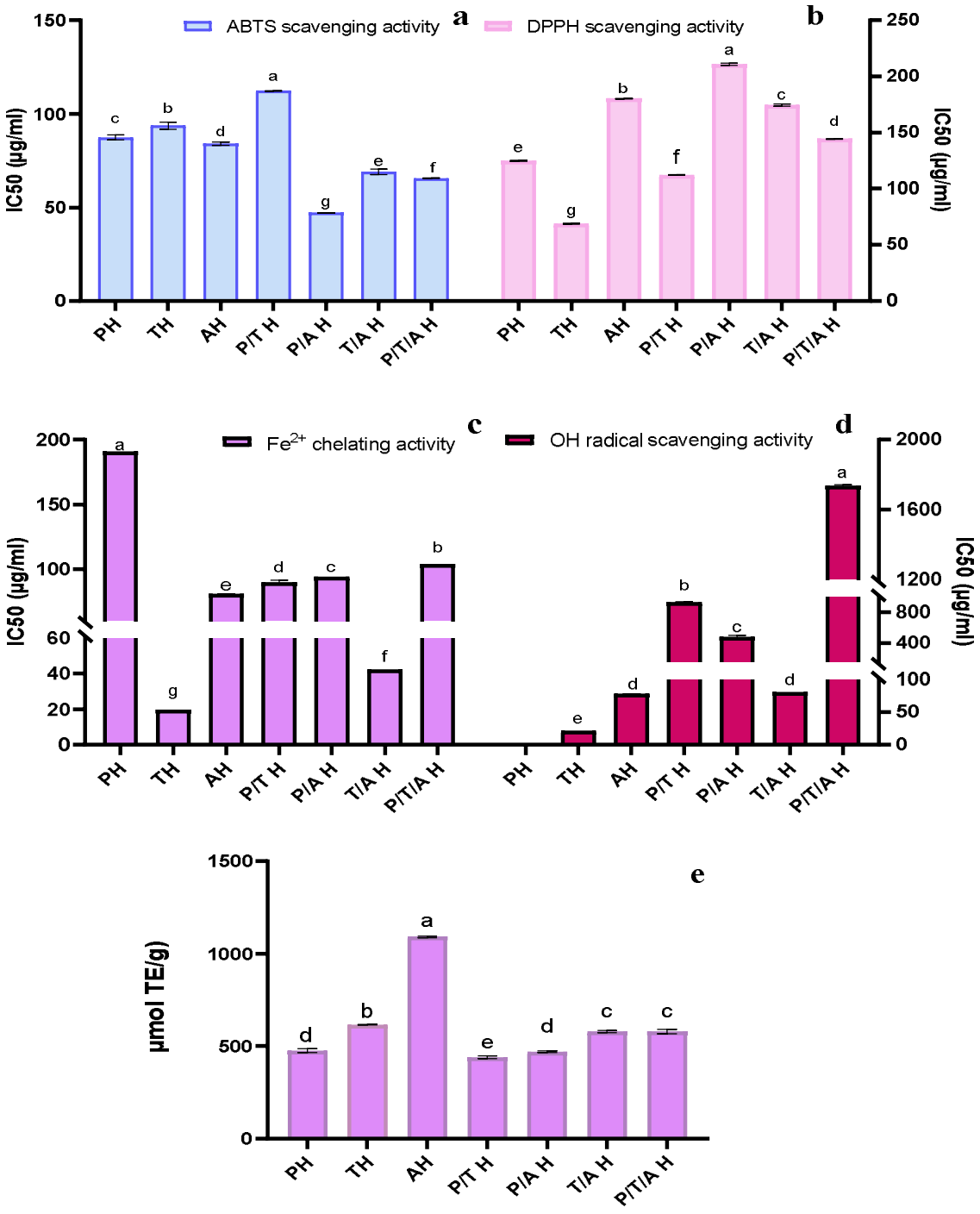


Figure 25: IC_{50} values of hydrolysates for ABTS radical scavenging activity (a), DPPH radical scavenging activity (b), Fe^{2+} chelating activity (c), OH radical scavenging activity (d), and ORAC values (µmol TE/g). Different letters (a–g) show significant differences ($p < 0.05$) between hydrolysates.

II.2.3.4. ORAC assay

ORAC is a widely used antioxidant assay that is based on the quantification of the emitted fluorescence from a probe, in this case, the protein fluorescein. The presence of AAPH exposes fluorescein to an oxidative environment, leading to protein degradation and subsequent loss of fluorescence. The ORAC assay measures the capacity of an antioxidant to neutralize free radicals through hydrogen donation, serving as an assessment of both general and specific antioxidant capabilities (Gillespie et al., 2007).

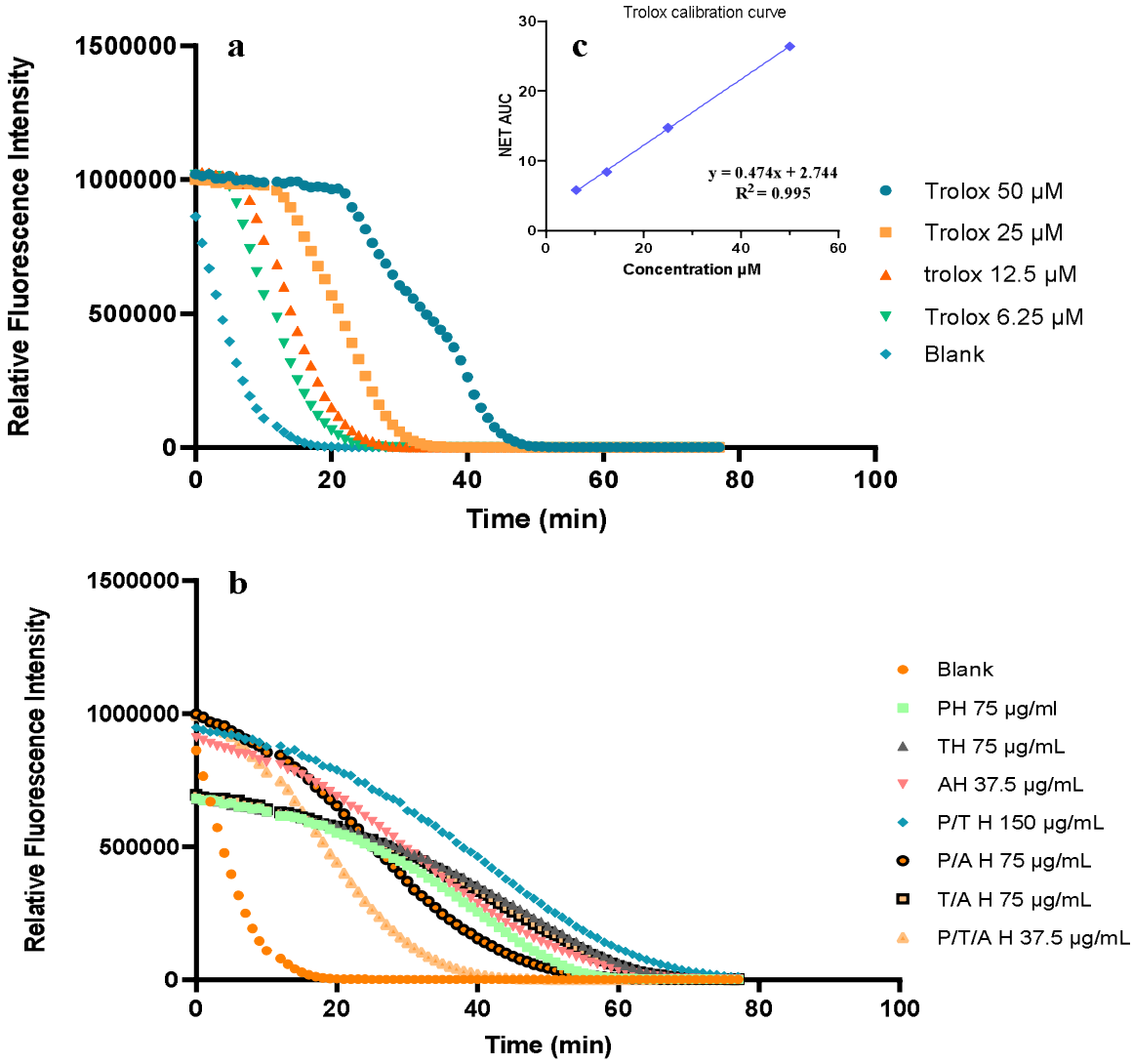


Figure 26: Fluorescence decay curves of Trolox (a) and hydrolysates (b). Regression of net AUC of Trolox (c) (net AUC = $AUC_{sample} - AUC_{blank}$).

Figure 26 demonstrates the fluorescence decay of protein hydrolysates over time. The ORAC value, expressed as µmol TE/g, of hydrolysates displayed significant variations ($p < 0.0001$) (Figure 26.e). Among all hydrolysates, AH exhibited the highest ORAC value (1090.491 ± 4.136 µmol TE/g). This result is similar to the reported value for peanut hydrolysate (1160 µmol TE/g) (Zheng et al., 2012), slightly lower than that of walnut hydrolysate (1752.98 µmol TE/g) (ref: walnut 2), and somewhat under that of chia seed hydrolysate (1535.81 µmol TE/g) (Urbizo-Reyes et al., 2019). The ORAC value of TH ranked second (615.773 µmol TE/g), while the ORAC values of the other hydrolysates ranged from 439.489 ± 7.66 µmol TE/g to 579.053 ± 11.15 µmol TE/g. The favorable ORAC values of the hydrolysates are likely attributed to the exposure of nonpolar residues during hydrolysis, such as Gly, Ala, Ile, Trp, Tyr, and Met. These residues can potentially enhance hydrophobic interactions between peptides and oxidizing agents (Marques et al., 2015).

II.2.3.5. Fe^{2+} chelating activity

Transition metal ions such as iron (Fe) and copper (Cu) prompt the generation of reactive oxygen species, leading to processes like lipid peroxidation and damage to DNA. Specifically, Fe^{2+} generates the hydroxyl radical through the Fenton reaction, which accelerates the chain reaction of lipid peroxidation. This reaction further breaks down lipid peroxides, resulting in the development of off-flavors. As a result, peptides binding to transition metal ions can retard this oxidation process (Najafian and Babji, 2014; Hu et al., 2018).

The ability of pine nuts protein hydrolysates to chelate Fe^{2+} is shown in Figure 24.d and figure 25.c. All protein hydrolysates demonstrated notable Fe^{2+} chelating activity. Notably, TH displayed a strong chelating activity with an IC_{50} value of 19.50 ± 0.008 ug/mL, followed by the chelating activity of T/A H (IC_{50} = 42.446 ± 0.028 µg/mL). On the other hand, PH exhibited the lowest chelating activity, recording an IC_{50} value of 190.90 ± 0.00 µg/mL. Peptides containing sulfur-containing amino acids like Cys and Met are generally responsible for the metal ion chelation capacity, reducing the pro-oxidant properties of some (Urbizo-Reyes et al., 2019). Additionally, the presence of aromatic rings in peptides may contribute to their enhanced metal chelation activity (Pownall et al., 2010).

II.2.3.6. FRAP

The assessment of reducing power is frequently utilized to measure the capability of antioxidants to donate electrons, thereby achieving the stabilization of free radicals (Sampath Kumar et al., 2012). The determination of the reducing power of various hydrolysates was conducted at 700 nm, and the results are illustrated in Figure 24.e. The reducing power of the hydrolysates was dose-dependent, with PH exhibiting the highest reducing power (OD_{700nm} = 1.227 ± 0.001 at 1069 µg/mL) among all hydrolysates, while AH exhibited the lowest activity. Peptides with good reducing power act by donating electrons to radicals, thereby stabilizing them, and preventing oxidative harm. Notably, the effectiveness of peptides in acting on radicals is influenced by their unique amino acid composition, structure, and concentration. Peptides containing amino acids known for their strong reducing attributes, such as Cys and Met, are especially proficient at donating electrons. Additionally, the presence of aromatic amino acids like Phe and Tyr can heighten the antioxidant potential of peptides owing to their capacity to effectively scavenge radicals. (Aluko and Aluko, 2012; Aondona et al., 2021).

II.2.4. HPLS-MS/MS

In order to identify the peptide sequence of hydrolysates demonstrating notable antioxidant, TH, T/A H, and P/T H were subjected to HPLC-MS/MS analysis. Subsequently, the acquired MS/MS spectra were matched to Pin p1 (main allergen in pine nuts) using database research software (Mascot, version 2.4). The software assigns a matching score to each identified peptide and a higher score indicates a more confident match between the observed MS/MS spectrum and a corresponding peptide sequence in the database.

Table XV: Peptide sequences identified by HPLC-MS/MS.

Protein hydrolysate	Rt (min)	Measured m/z	Calculated Mw (Da)	range	Peptide sequence	
					One-letter code	Three-letters code
TH	26.179	842.291	841.309	92 - 98	DQSQSYD	Asp-Gln-Ser-Gln-ser-Tyr-Asp
	21.962	748.294	747.340	90 - 96	ALDQSQS	Ala-Leu-Asp-Gln-Ser-Gln-Ser
	27.657	828.401	827.414	133 - 139	AEELPNR	Ala-Gly-Glu-Leu-Pro-Asn-Arg
	30.786	694.285	693.345	58 - 62	DYLQR	Asp-Tyr-Leu-Gln-Arg
	36.348	674.180	1345.611	124 - 134	REEEEAVERAE	Arg-Glu-Glu-Glu-Glu-Ala-Val-Glu-Arg

	16.969	533.100	532.249	90 - 94	ALDQS	Ala-Leu-Asp-Gln-Ser
P/T H	73.498	351.856	702.341	120 - 125	EGRGRE	Glu-GlyArg-Gly-Arg-Glu
	42.229	540.130	539.241	1 - 5	MGVFS	Met-Gly-Val-Phe-Ser
	14.635	674.259	673.339	73 - 77	EELQR	Glu-Glu-Leu-Gln-Arg
	26.933	828.387	827.414	133 - 139	AEELPNR	Ala-Glu-Glu-Leu-Pro-Asn-Arg
	7.099	745.279	744.34	65 - 70	EQPSER	Glu-Gln-Pro-Ser-Glu-Arg
	6.565	745.292	744.34	65 - 70	EQPSER	Glu-Gln-Pro-Ser-Glu-Arg
	30.497	694.293	693.345	58 - 62	DYLQR	Asp-Tyr-Leu-Gln-Arg
T/A H	19.896	758.277	757.459	19 - 25	ALLSLLE	Ala-Leu-Leu-Ser-Leu-Leu-Glu
	27.036	828.42	827.414	133 - 139	AEELPNR	Ala-Glu-Glu-Leu-Pro-Asn-Arg
	31.262	694.271	693.345	58 - 62	DYLQR	Asp-Tyr-Leu-Gln-Arg
	8.184	745.267	744.34	65 - 70	EQPSER	Glu-Gln-Pro-Ser-Glu-Arg
	8.659	745.309	744.34	65 - 70	EQPSER	Glu-Gln-Pro-Ser-Glu-Arg
	10.363	601.091	600.275	134 - 138	EELPN	Glu-Glu-Leu-Pro-Asn
	32.219	694.273	693.345	58 - 62	DYLQR	Asp-Tyr-Leu-Gln-Arg
	25.937	757.35	756.377	134 - 139	EELPNR	Glu-Glu-Leu-Pro-Asn-Arg

Table XV showcases the peptide sequences identified for each hydrolysate. Within TH, recognized for its potent ABTS radical scavenging, Fe^{2+} chelating, OH radical scavenging, ORAC, and DPPH activities, five distinct peptides emerged. Their molecular weights (Mw) ranged from 743.340 Da to 1345.61 Da. The observed antioxidant efficacy of TH could be attributed to the presence of hydrophobic amino acids such as Ala, Leu, and Tyr in peptides ALDQSQR, AEELPNR, and DYLQR. Notably, Tyr is acknowledged for its robust radical scavenging role due to the phenolic hydroxyl group (-OH) within its side chain, which functions as a radical scavenger.

For P/T H, three distinct peptides (ALDQS), (EGRGRE), and (MGVFS) were identified, exhibiting Mw values of 532.248 Da, 702.341 Da, and 539.241 Da, respectively. The presence of Met and Phe was detected in the sequence MGVFS, potentially contributing to P/T H's antioxidant activity. Met's recognized potent antioxidant ability is attributed to its sulfur atom in the side chain, facilitating electron donation to radicals. Furthermore, Phe, being an aromatic amino acid, adds to the antioxidant activity. However, P/T H's antioxidant effectiveness was not as potent, possibly due to the lower concentration of Met and Phe.

Conversely, T/A H manifested thirteen distinctive peptides, with Mw from 600.275 Da to 827.414 Da (Table XV). Interestingly, Four EQPSER peptides, and three DYQLR peptides were identified within the composition of T/A H. The good antioxidant activity of T/A H may arise from the collective interaction of various amino acids within its composition.

II.3. Conclusion

The aim of this study was to produce diverse hydrolysates with unique characteristics in terms of bioactivity and peptide composition through the use of different enzymes, both individually and sequentially. The results revealed notable variations between the protein hydrolysates generated in terms of bioactivity and the peptide sequences they contained. Remarkably, all hydrolysates displayed distinguished antioxidant activity, with TH, in particular, demonstrating the most pronounced bioactivity across various test measurements. In the light of these results, it can be deduced that proteins derived from pine nuts show promising potential as a source for the production of peptides with antioxidant properties.

Chapter III. Immunoreactivity of pine nuts (Pinus pinea L.) protein isolate and trypsin hydrolysate and the effects of simulated in vitro gastrointestinal digestion on the antioxidant activity of trypsin hydrolysate

Chapter III. Immunoreactivity of pine nuts (*Pinus pinea* L.) protein isolate and trypsin hydrolysate and the effects of simulated *in vitro* gastrointestinal digestion on the antioxidant activity of trypsin hydrolysate

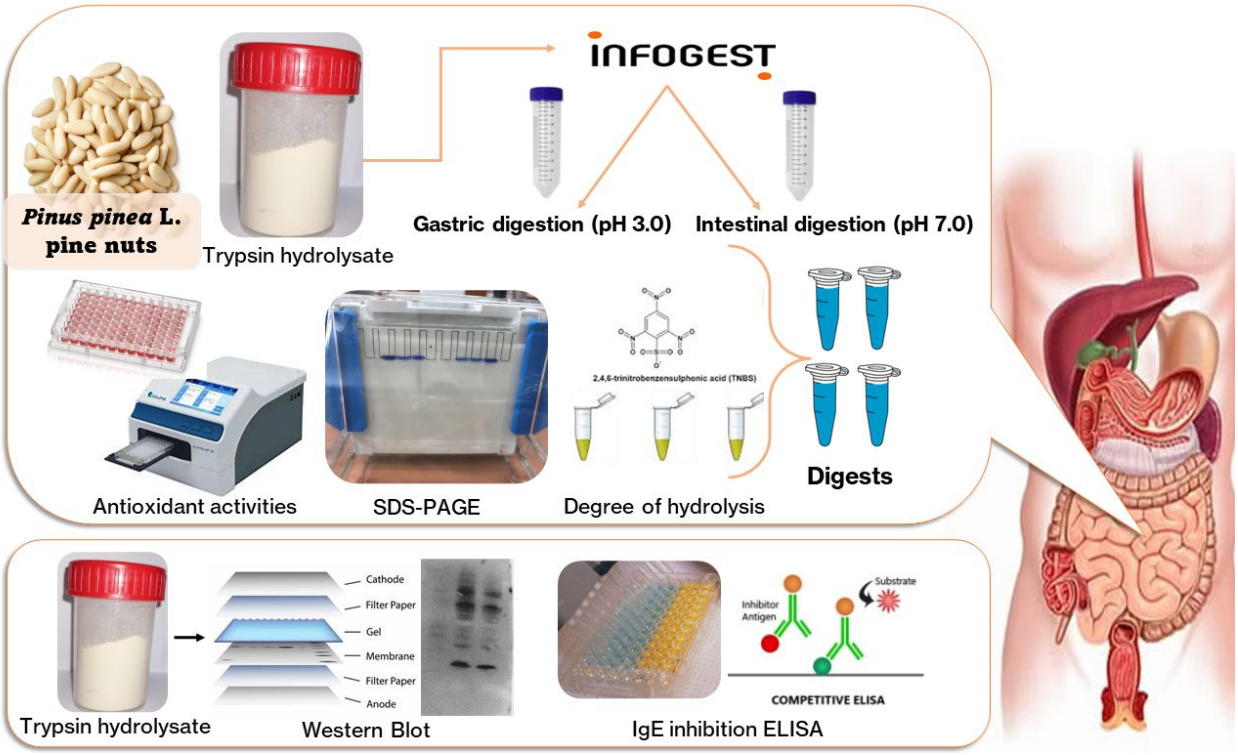


Figure 27: Graphical abstract representing the simulated gastrointestinal digestion of trypsin hydrolysates, the assessment of the antioxidant activity of the digests, their SDS-PAGE analysis, and the assessment of the immunoreactivity of trypsin hydrolysate.

Abstract

One of the primary challenges in the development of nutraceuticals and functional food ingredients is their bioavailability. The objective of this study is to assess the impact of the simulated *in vitro* gastrointestinal digestion on the antioxidant capabilities of trypsin hydrolysate. This will be evaluated using various assays including DPPH Radical, ABTS radical, and OH radical scavenging activities, as well as Fe^{2+} chelating activity, and ORAC and FRAP assays. Additionally, the second part of the study involved analyzing the immunoreactivity of PI and TH, through western blot and inhibition ELISA, to pine nut-allergic individuals' sera. When subjected to simulated gastrointestinal digestion, different electrophoretic profiles of TH and control samples (PI and PIL) were observed by SDS-PAGE analysis. Findings demonstrate that the antioxidant activities of ABTS radical scavenging, Fe^{2+} chelation, and ORAC were enhanced by the simulated digestion process for TH. Meanwhile, its ferrous reducing power remained consistent. Nevertheless, the scavenging abilities against DPPH and OH radicals were diminished, yet the potency of the activity persisted. Immunoreactivity testing revealed that PI was immunoreactive with IgE from the sera of pine nut-allergic individuals. Conversely, for TH, immunoreactivity was reduced, indicating that the enzymatic hydrolysis of pine nut proteins by trypsin has effectively reduced their allergenic potential.

Keywords: Pine nut trypsin hydrolysate, Simulated *in vitro* gastrointestinal digestion, antioxidant activity, immunoreactivity.

III.1. Materials and methods

III.1.1. Preparation of PI

In this section, a different method was employed to prepare the protein isolate (PI), differing from the technique utilized in the earlier study (I.1.2.2). The process involved dissolving defatted pine nut flour in alkaline MilliQ water (pH 8). After thorough vortexing, the mixture was then centrifuged for 15 min at 8000 × g. The resulting supernatant was collected and subjected to freeze-drying. The final protein content of this particular sample is measured at 85.89 ± 0.060%.

III.1.2. Preparation of trypsin hydrolysate (TH)

In the previous part (part III, chapter II), TH displayed the most effective antioxidant properties. In the current experiment, TH was prepared following the procedure outlined in the preceding section (II.1.2.2.1). In summary, a suspension of PI was prepared in 50 mM phosphate buffer, pH 8 at a concentration of 5 mg/mL, and then heated to 37°C for 10 minutes. The pH was readjusted to 8.0, and trypsin from bovine pancreas (with a trypsin activity of 147.274 U/mg) was introduced at an E/S ratio of 0.377 U/mg of protein. The hydrolysis process took place over 4 h at 37°C, with intermittent pH monitoring. Once hydrolysis was complete, the enzymatic action was halted by subjecting the sample to a temperature of 85 °C for 15 min, using a water bath. Subsequently, the samples were centrifuged at 8000 × g for 15 min at 4°C. The resulting supernatants were collected, freeze-dried, and stored at -20°C for subsequent analysis.

III.1.3. *In vitro* simulated gastrointestinal digestion

TH was subjected to the *In vitro* simulated gastrointestinal digestion, along with PI and wholemeal pine nut flour (PIL), which were used as control samples.

In the process of preparing the *in vitro* digestion, determining the activity of digestive enzymes and the concentration of bile salts is a crucial phase. This determination needs to be carried out experimentally for each fresh batch of enzymes or after extended storage periods. The enzymatic activities of both gastric and pancreatic pepsin, lipase, and trypsin were evaluated using the methods outlined by Minekus et al. (2014).

III.1.3.1. Enzyme assays

III.1.3.1.1. Pepsin activity assay

The pepsin activity of both pepsin from porcine gastric mucosa and rabbit gastric extract (RGE) was assessed according to Minekus et al. (2014). In Eppendorf tubes, 500 µL of 2% (w/v)

bovine blood hemoglobin (pH 2) were introduced, and the tubes were preincubated in a shaking incubator at 37°C for a span of 4 minutes. Subsequently, 100 µL of pepsin and RGE solutions, having varying concentrations, were added to the mixture, which was then incubated for precisely 10 minutes. To halt the reaction, 1000 µL of 5% TCA were added, followed by centrifugation at 6000 × g for 30 min to precipitate hemoglobin. The resulting supernatant was transferred to a quartz cuvette, and the absorbance was measured at 280 nm. The same procedural steps were executed for the blank, except that Pepsin or RGE was introduced after the addition of TCA. Pepsin activity was determined using the subsequent formula:

$$\text{Pepsin activity (U/mg)} = (A_{280}\ \text{sample} - A_{280}\ \text{blank}) / (\Delta t \times X)$$

Where, Δt signifies the reaction time (10 minutes), and X represents the concentration of pepsin or RGE in the final reaction mixture (mg/mL).

III.1.3.1.2. Pancreatic trypsin activity assay

Pancreatic trypsin activity was assessed through the introduction of a particular mixture into a quartz cuvette according to Minekus et al. (2014) protocol. This mixture comprised 1300 µL of 46 mM Tris/HCl buffer (pH 8.1) and 150 µL of 10 mM p-Toluene-Sulfonyl-L-arginine methyl ester (TAME), a substrate specific to trypsin. The mixture was mixed through inversion and then placed in UV-visible spectrophotometer, incubated at a 25°C for a duration of 3 min. Afterward, 50 µL of pancreatin from porcine pancreas, prepared at various concentrations, were added to the aforementioned mixture. The subsequent increase in absorbance was continuously monitored at 247 nm for a span of 10 minutes, until a steady level was achieved. Comparable procedures were employed for generating a blank sample, with the exception that no enzyme was added. The pancreatic trypsin activity was calculated using the following formula:

$$\text{Trypsin activity (U/mg)} = (\Delta_{247}\ \text{sample} - \Delta_{247}\ \text{blank}) * 1000 * 1.5 / (540 \times X)$$

Where, Δ_{247} signifies the slope of the initial linear segment of the curve (U/min), 540 represents the molar extinction coefficient of TAME at 247 nm (L / (mol × cm)), and 1.5 accounts for the final volume of the reaction mixture (mL).

III.1.3.1.3. Pancreatic and gastric lipase activity assay

The lipase activity of pancreatin from porcine pancreas and that of RGF was determined through titration, utilizing tributyrin as the substrate (Minekus et al., 2014). The assay solutions were prepared according to the details provided in Table XVI. In a titration vessel, a total of 7250 µL of assay solution was combined with 250 µL of tributyrin, mixed, and allowed to stabilize at a

temperature of 37°C. Subsequently, 50 µL of pancreatin/RGE solution was introduced into the mixture. A titrant solution (0.1 N NaOH) was added gradually, while closely monitoring the precise volume required to maintain pH 8 (for pancreatin) or pH 5.5 (for RGE) over a span of 5 minutes.

Table XVI: Preparation of assay solutions, the quantities are computed for a final volume of 200 mL.

Assay solution components	RGE lipase activity	Pancreatic lipase activity
tris-(hydroxymethyl)-aminomethane (1.48 mM)	-	7.20 mg
Sodium taurodeoxycholate	215.08 mg (24 mM)	420 mg (4 mM)
NaCl (150 mM)	1800 mg	1800 mg
$CaCl_2$ (1.4 mM)	-	40 mg
BSA (1.5 µM)	0.019 mg	-

The lipase activity of pancreatin and RGE was computed as outlined below:

- **For pancreatic lipase**

$$R\ (NaOH)\ (\mu mol/min) = (\Delta v\ NaOH / (t_1\text{-}t_2)) \times N$$

$$Lipase\ activity\ (U/mg) = R\ (NaOH) \times 1000 / v \times C$$

- **For Rabbit gastric lipase**

$$R\ (NaOH)\ (\mu mol/min) = (\Delta v\ NaOH / (t_1\text{-}t_2)) \times N \times 1.12$$

$$Lipase\ activity\ (U/mg) = R\ (NaOH) \times 1000 / v \times C$$

Where, Δv NaOH represents the volume of NaOH introduce (µL), during the interval between times t_2 and t_1. N denotes the normality of NaOH (0.1 N), v signifies the volume of the enzyme solution added (µL), C stands for the concentration of the enzyme, and 1.12 serves as a correction factor used to attain the complete activity.

III.1.3.1.4. Determination of bile acid concentration

The bile acid concentration was determined in accordance with the bile acid assay kit's technical bulletin. In summary, for the sample, blank, and standard wells in a black 96-well plate with a clear bottom, 20 µL of bovine bile extract (0.8 mg/mL in MilliQ water) was added. Following this, in the sample wells, 5 µL of MilliQ water and 80 µL of working reagent were introduced. The working reagent comprises 150 µL of assay buffer along with 16 µL of NAD, 8

µL of Probe, 2 µL of enzyme A, and 2 µL of enzyme B.

For the standard sample, the wells containing bovine bile sample were supplemented with 5 µL of internal standard (80 µM sodium cholate, prepared by combining 4 µL of standard and 46 µL of MilliQ water), followed by the addition of 80 µL of working reagent.

In the case of the blank sample, 5 µL of MilliQ water was added to the bovine bile sample, and then blank reagent was added, containing 150 µL of assay buffer, 16 µL of NAD, 8 µL of Probe, and 2 µL of enzyme B.

The plate was tapped and incubated for 20 min in the dark. Subsequently, the fluorescence intensity was measured at an excitation wavelength of 530 nm and an emission wavelength of 585 nm. The calculation of bile acid concentration was carried out using the following formula:

$$\text{Bile acid concentration } (\mu M) = (F_{sample} - F_{blank}) / (F_{standard} - F_{sample}) \times 20 \times n$$

Where 20 represents the effective concentration of the internal standard, and n signifies the dilution factor.

III.1.3.2. Simulated gastrointestinal digestion of TH, PI, and PIL

The *in vitro* gastrointestinal digestion of TH, PI, and PIL was performed according to the revised INFOGEST 2.0 protocol (Brodkorb et al., 2019), which replicates the physiological conditions of human digestion in two phases – the gastric and intestinal phases. For each substance, TH, PI, and PIL, solutions were prepared at a concentration of 50 mg/mL in simulated gastric fluid (SGF). These solutions were combined with SGF in a 50:50 ratio (v/v), and after adding additional components and water, the pH was adjusted to 3.0 with 1 M HCl. Pepsin from porcine gastric mucosa (pepsin activity of 3580.7 U/mg) was added to achieve a concentration of 2000 U/mL in the final digestion mixture. RGE (lipase activity of 110.75 U/mg) was added to PIL based on its gastric lipase activity to achieve 60 U/mL in the final digestion mixture. The mixtures were then placed in a shaking incubator at 37°C for 2h with continuous agitation. Samples were taken during the gastric digestion at specific intervals (0, 10, 20, 30, 60, and 120 min), and the pH was adjusted to 7.0 using 1 M $NaHCO_3$ to halt the pepsin activity.

Following the 2-hour gastric digestion, the digested samples were blended with simulated intestinal fluid (SIF) in a 50:50 ratio (v/v). The pH was raised to 7 using 1 M NaOH, and a solution of 300 mM $CaCl_2$ was introduced to achieve a final concentration of 0.075 mM. Pancreatin from porcine pancreas (Trypsin activity of 0.735 U/mg) was then added to TH, PI, and PIL mixtures

based on its a trypsin activity to achieve a concentration of 100 U/mL in the ultimate digestion mixture. Subsequently, bile bovine extract (with a bile acid concentration of 3572 µM) was added to reach a final concentration of 10 mM in the digestion mixture. The mixtures were incubated at 37°C with continuous shaking for another 2 hours, representing the intestinal digestion phase. Sampling took place at specific time intervals during the intestinal digestion (0, 10, 20, 30, 60, and 120 min) and the reaction was terminated by adding Bowman-Birk protease inhibitor. All samples were thoroughly mixed and then stored at -20°C for subsequent analysis.

III.1.4. Determination of DH

The degree of hydrolysis (DH) of the simulated gastrointestinal digests of TH, PI, and PIL was assessed using the procedure outlined in the previous section (II.1.4).

III.1.5. SDS-PAGE analysis

The SDS-PAGE of TH, PI, and PIL, along with their gastrointestinal digests, was performed following the procedures outlined in section II.1.5.

III.1.6. Evaluation of Antioxidant activity

The DPPH radical scavenging activity, ABTS radical scavenging activity, OH radical scavenging activity, Fe^{2+} chelating activity, ferrous reducing power capacity, and ORAC were employed to assess the antioxidant activity of TH and PI gastrointestinal digests, following the methods detailed in section II.1.6.

III.1.7. *In vitro* assessment of allergenic reactivity

III.1.7.1. Specific IgE detection by western blotting

TH, PI, and PIL samples separated using SDS-PAGE were electrophoretically transferred onto an Amersham hybond P 0.45 polyvinylidene difluoride (PVDF) blotting membrane. Subsequently, the membrane was rinsed with Tris-buffered saline containing 0.05% Tween-20 (TBST-0.05%) and then blocked with 1% BSA overnight at 4°C, employing gentle agitation. After this, the membrane was washed twice with TBST-0.05% and left to incubate overnight at 4°C with a pool of sera prepared from sera sourced from 4 pine nuts-allergic individuals, using a dilution ratio of 1:300. Following another washing step, the membrane was subjected to a 4-hour incubation with a 1:500 dilution of Anti-IgE-biotin secondary antibody, which had been prepared in TBST-BSA-0.1%. Subsequently, the membrane was once again washed and exposed to Streptavidin-HRP at a dilution of 1:3000, with an incubation period of 1h at room temperature.

After undergoing six washes with TBST-0.05%, the detection of IgE-binding components was achieved through enhanced chemiluminescence, utilizing an ECL substrate as per the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ, USA) (Benedé et al., 2014).

III.1.7.2. IgE ELISA inhibition

To evaluate the inhibition of IgE-binding in TH, an inhibition ELISA method was employed using a pool of sera collected from four pine nut-allergic patients as described by Benedé et al. (2014). Polystyrene 96-well microtiter plate was coated with 50 µL/well of PI (antigen) at a concentration of 50 µg/mL. This plate was then allowed to incubate overnight at 4°C. Following this, the wells were rinsed with PBS containing 0.05% of Tween-20 (PBST-0.05%) and subsequently blocked using a blocking solution consisting of PBS with 2.5% of Tween-20, for a duration of 4h.

The pool of sera (diluted 1:200) was preincubated at room temperature for 2h with equivalent volumes of TH samples and PI (control), both prepared at varying concentrations. After another round of washing, the pre-incubated mixtures of pool of sera and samples were introduced into the wells and allowed to incubate for 2h. After washing the wells again, an Anti-IgE-Biotin secondary antibody, diluted at 1:1000, was added, and the microplate was incubated for 1h. After this step, Avidin-HRP (diluted 1:10,000) was added to the wells and incubated for 30 minutes.

Following another round of washing, a peroxidase substrate called tetramethylbenzidine (TMB) was introduced at 50 µL/well to initiate the peroxidase reaction. The reaction was halted using 0.5 M H_2SO4, and the resulting optical density was measured at 450 nm using a microplate reader (Multiskan FC Microplate Photometer, Thermo Fisher Scientific Inc., MA, USA).

III.1.8. Statistical analysis

All experiments were performed in triplicate (n = 3) and expressed as mean ± SD. A one-way analysis of variance (ANOVA), using GraphPad Prism software version 8.0.2, was performed to compare mean values. Significant differences between sample means and those of the control group (THC, PIC, and PILC) were evaluated using Dunnett's multiple comparisons test ($p < 0.05$).

III.2. Results and discussion

III.2.1. DH of the gastrointestinal digests

As presented in Figure 28, the degree of hydrolysis (DH) was 2.73% for PI, 1.03% for PIL, and 6.234% for TH (at 0 min). After a 120-minute gastric digestion employing pepsin (for PI and

TH) and pepsin combined with RGE (for PIL), the DH values increased to 4.41% (PI), 9.788% (TH), and 6.92% (PIL). Following an additional 120-minute incubation with pancreatin (intestinal digestion), a notable increase in DH was observed, reaching 74.17% for TH, 80.288% for PIL, and 98.84% for PI.

This disparity suggests that pepsin and RGE resulted in the breakdown of fewer peptide fragments compared to pancreatin. Pepsin, as an endopeptidase, primarily cleaves the C-terminal of Phe, Tyr, and Trp amino acids, likely leading to the fragmentation of peptides into smaller entities. In contrast, pancreatin encompasses a variety of gastrointestinal enzymes, including trypsin, amylase, lipase, ribonuclease, and protease, which collectively enhance the efficiency of polypeptide cleavage. This implies that peptides have undergone hydrolysis into even smaller peptide fragments (oligopeptides) or have been completely degraded into individual amino acids (Xiao et al., 2014).

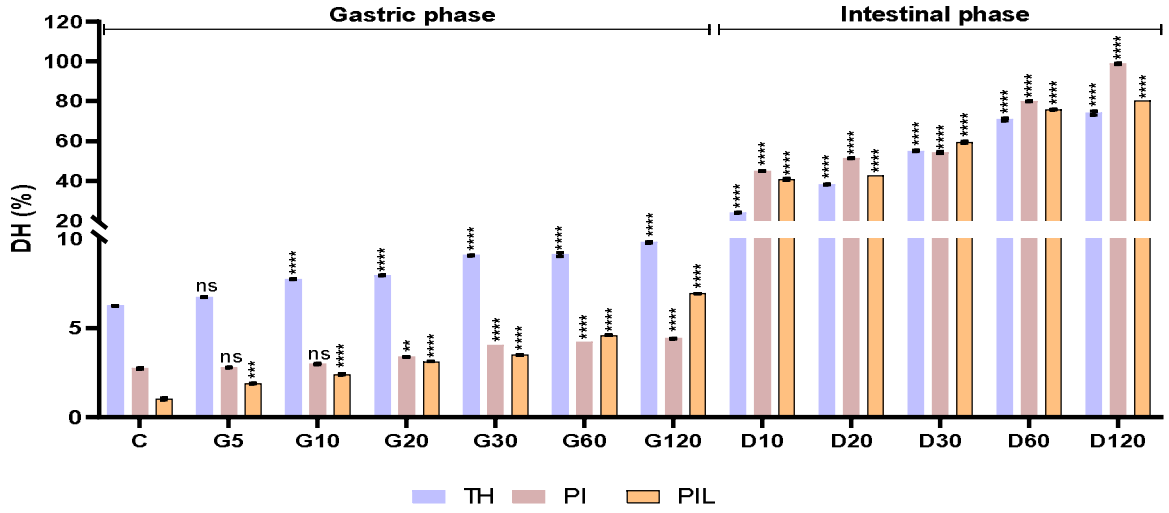
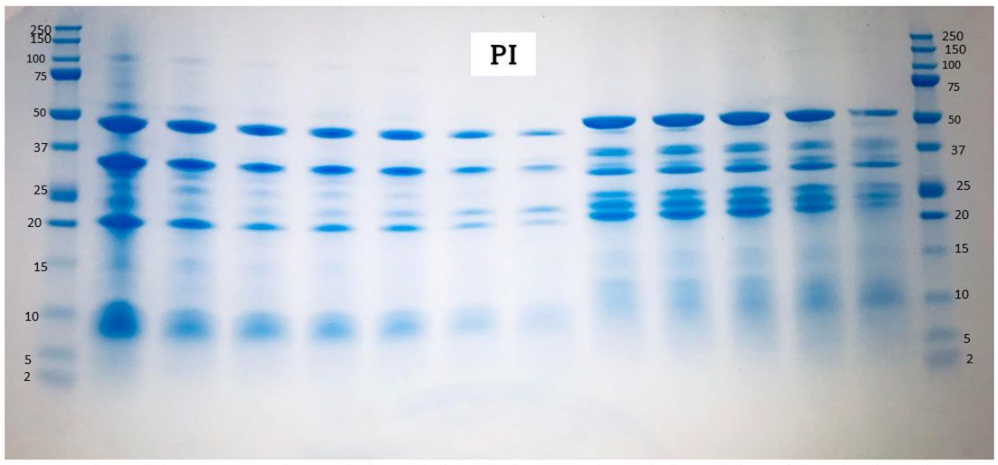
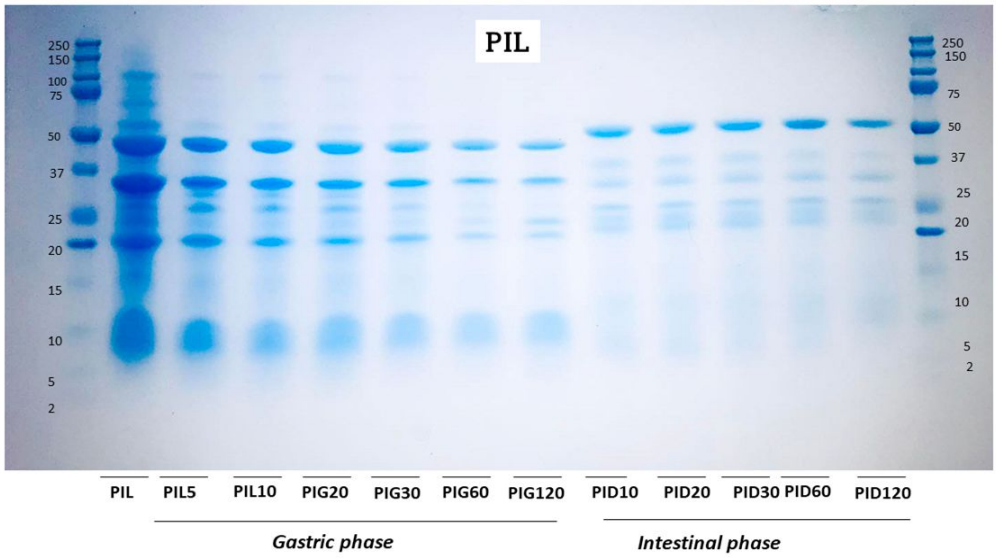


Figure 28: Degree of hydrolysis (%) of TH, PI, PIL digests throughout gastrointestinal digestion process. Values are expressed as mean ± SD, n = 3. ns = non-significant; **p < 0.01; ***p < 0.001; ****p < 0.0001 compared with the control group (THC, PIC, and PILC).

III.2.3. SDS-PAGE analysis of simulated gastrointestinal digestates

Under reducing conditions, the SDS-PAGE analysis of pine nut proteins (PI and PIL) exhibited four noticeable bands around 49, 36, 20, and 6-11 kDa, accompanied by smaller, less prominent bands approximately at 25, 27, and 34 kDa, forming a diffuse pattern. Additionally, two minor bands were observed at roughly 100 kDa and 51 kDa, while PIL displayed a significant


PI
250
150
100
75
50
37
25
20
15
10
5
2
PIC
PIG5
PIG10
PIG20
PIG30
PIG60
PIG120
PID10
PID20
PID30
PID60
PID120
Gastric phase
Intestinal phase


PIL
250
150
100
75
50
37
25
20
15
10
5
2
PIL
PIL5
PIL10
PIG20
PIG30
PIG60
PIG120
PID10
PID20
PID30
PID60
PID120
Gastric phase
Intestinal phase

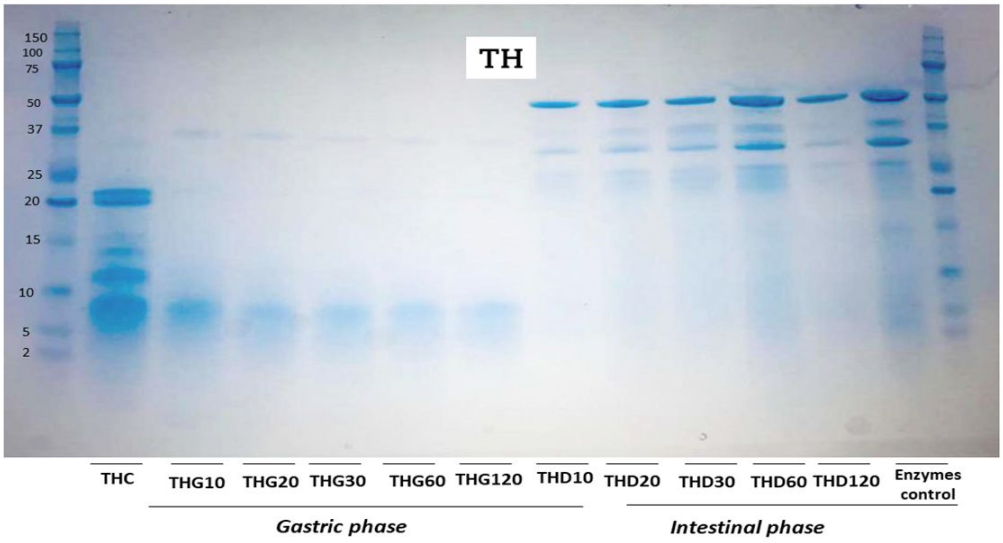

TH
150
100
75
50
37
25
20
15
10
5
2
THC
THG10
THG20
THG30
THG60
THG120
THD10
THD20
THD30
THD60
THD120
Enzymes control
Gastric phase
Intestinal phase

Figure 29: SDS-PAGE gels display the effect of *in vitro* gastrointestinal digestion on TH, PI, and PIL. Serving as controls, undigested samples are labeled as THC, PIC, and PILC. Digests from TH, PI, and PIL during gastric digestion at varying time intervals are denoted as THG (10-120), PIG (5-120), and PILG (5-120). Correspondingly, the digests obtained during intestinal digestion are labeled as THD (10-120), PID (5-120), and PILD (5-120), also representing different time intervals in minutes.

band at 2 kDa. A prior study noted the revelation of four prominent bands in pine nut proteins with molecular weights at 46, 31, 23, and 10-17 kDa (Ma et al., 2010).

In the case of TH, the electrophoretic analysis under reducing conditions demonstrated four noticeable bands at 21, 20, 11, and 5-10 kDa, alongside two smaller bands at 14 and 16 kDa.

Figure 29 depicts the SDS-PAGE analysis of PI, PIL, and TH subjected to simulated gastrointestinal digestion. For PI and PIL, specific protein bands at 49, 36, 20, and 6-11 kDa remained unchanged during the initial 0-30 minutes of pepsin digestion. From 30 to 120 minutes of pepsin digestion, these bands underwent partial hydrolysis. Conversely, bands at 100, 51, 25, 27, and 34 kDa were entirely hydrolyzed.

The intensity of the Pin p1 band (6-11 kDa) diminished over time, indicating its partial hydrolysis by pepsin. Similar observations were documented concerning pine nut proteins, where bands at 46, 31, and 23 kDa demonstrated stability under pepsin digestion for 0-120 minutes, while the 10-17 kDa band displayed partial hydrolysis right from the start of simulated gastric conditions, ultimately appearing as a 10-15 kDa band (Toomer et al., 2013)**.**

During the simulation of intestinal digestion, the protein profile of the pancreatin enzyme was analyzed for reference (Figure 29, TH). The analysis of the pancreatin protein profile identified bands with molecular weights around 50, 37, 35, and 25 kDa, along with minor bands in the 20 to 24 kDa range that appeared as a blurred pattern. Recognizing that these bands were attributed to pancreatin, they were excluded from the assessment of the protein profiles of pine nut protein samples.

In the cases of PI and PIL, the 49 kDa band emerged as a faint presence at 10, 20, and 30 min during the intestinal digestion phase, and by 60 min, it had undergone full hydrolysis. Similarly, the 36 kDa band maintained its faint nature from the outset to 120 min into the intestinal digestion. Intriguingly, the Pin p1 band experienced a higher degree of hydrolysis compared to the gastric phase, yet it remained partially intact, presenting itself as a band with a molecular weight ranging from 10 to 11 kDa.

During the simulated intestinal digestion of TH samples, the residual Pin p1 band that had persisted from the gastric digestion underwent complete hydrolysis. This transformation indicates that the Pin p1 band had been broken down into smaller peptide fragments (such as di- or tripeptides) as well as individual free amino acids.

III.2.4. Antioxidant activities

Antioxidant hydrolysates and peptides should maintain their activity following enzymatic digestion to exert their effects within the body. This study aims to assess the influence of simulated *in vitro* gastrointestinal digestion on the antioxidant characteristics of TH and PI using various assays.

III.2.4.1. DPPH radical scavenging activity

DPPH radical, a stable free radical commonly used to assess the antioxidant efficacy of natural compounds, served as a testing parameter. The IC_{50} values for PI (used as a control) and TH digests were computed and are graphically depicted in Figure 30.

During gastrointestinal digestion, the scavenging activity exhibited significant fluctuations due to varying incubation times with pepsin and pancreatin. Notably, these changes weren't consistent over time, indicating that distinct peptides with diverse antioxidant properties were generated as the samples interacted with digestive enzymes minute by minute. Following a 5-minute simulated gastric digestion using pepsin, a notable reduction in the scavenging activity of TH was observed (IC_{50} decreased from 111.57 ± 1.006 to 946.94 ± 49.65 µg/mL). Subsequently, activity displayed an increase.

The highest scavenging activity for TH was recorded during intestinal digestion at the 20-minute mark (THD20) with an IC_{50} of 90.66 ± 0.394 µg/mL. Following this, a gradual decrease in activity occurred, resulting in an IC_{50} of 247.613 ± 0.921 µg/mL at 120 minutes (THD120). This decrease implies that during intestinal digestion with pancreatin, TH peptides were fully hydrolyzed, potentially leading to the accumulation of shorter peptides like di- and tripeptides as well as amino acids. Consequently, the digests acquired heightened hydrophilicity. The elevated polarity of the digestates renders them less reactive towards lipid-soluble DPPH radicals, thus explaining the diminished radical scavenging activity post-pancreatin treatment (Zhu et al., 2008; You et al., 2010a).

Following simulated gastric digestion, a sharp increase in PI's activity was evident (IC_{50} decreased from 1903.77 ± 99.61 to 477.930 ± 39.498 µg/mL), indicating that pepsin had liberated antioxidant peptides from PI. Subsequent digestion with pancreatin yielded a generation of antioxidant peptides, and the most potent activity was observed in PID30 (IC_{50} = 436.08 ± 54.238 µg/mL). However, a subsequent decrease in activity transpired towards the conclusion of intestinal digestion (at 60 and 120 min). At these points, the degree of hydrolysis reached around 79.953% and 98.843%, respectively, indicating complete hydrolysis of peptides. These peptides were likely converted into di- or tripeptides, or even free amino acids, characterized by a hydrophilic nature that diminished their interaction with lipid-soluble DPPH radicals. Nevertheless, by the end of the intestinal digestion, the activity of PI surpassed that of undigested PI, signifying a notable enhancement in its antioxidative potential.

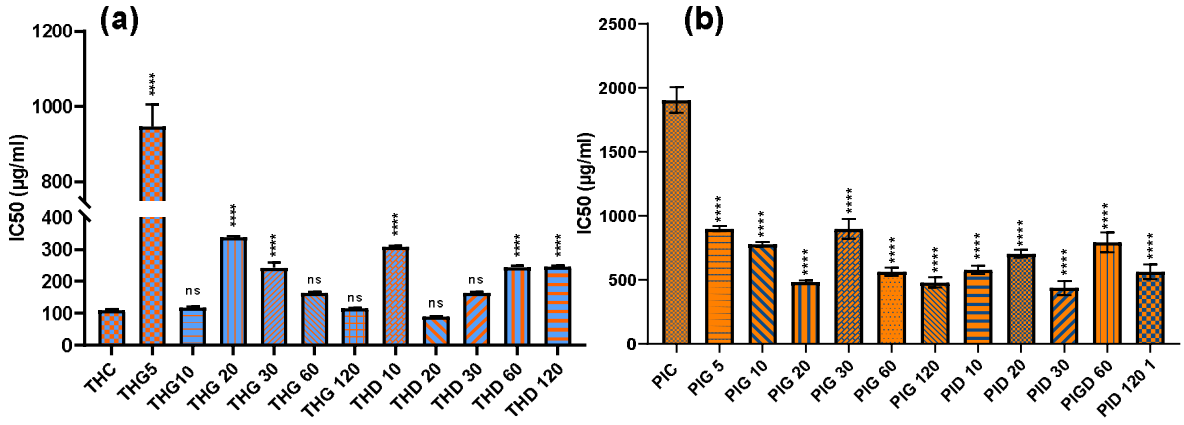


Figure 30: IC_{50} values (µg/mL) of TH digests (a) and PI digests (b) for DPPH scavenging activity. Values are expressed as mean ± SD, n = 3. ns= non-significant; ****p < 0.0001 compared with the control group (THC and PIC).

III.2.4.2. $ABTS^{\bullet+}$ radical scavenging activity

$ABTS^{\bullet+}$ represents a water-soluble radical cation that can be effectively scavenged by antioxidants within aqueous systems, leading to a decline in absorbance at 734 nm. The IC_{50} values for $ABTS^{\bullet+}$ radical scavenging activity of TH and PI digests are displayed in Figure 31.

During gastric digestion, the scavenging activity of TH experienced a reduction, shifting from IC_{50} = 331.232 ± 53.018 µg/mL for THC to 403.179 ± 56.334 µg/mL for THG120.

Conversely, for PI, a significant increase ($p < 0.0001$) was observed, transitioning from IC_{50} = 3333.74 ± 432.326 µg/mL to 1267.861 ± 140.720 µg/mL. Subsequent exposure to pancreatin yielded a noteworthy elevation ($p < 0.0001$) in scavenging activity for both PI and TH, with IC_{50} values of 151.186 ± 18.572 µg/mL and 53.571 ± 3.190 µg/mL, respectively.

The alteration in activity observed, decreasing during the gastric phase and increasing during the intestinal phase, can be attributed to the heightened hydrophobic attributes of TH digestates during the gastric phase. Unlike the DPPH radical, the $ABTS^{\bullet+}$ radical is water-soluble, potentially leading to reduced reactivity between TH digests with hydrophobic traits and the water-soluble $ABTS^{\bullet+}$ radical. Conversely, the augmentation of hydrophilic qualities in digests following pancreatin treatment has facilitated their interaction with and capture of the $ABTS^{\bullet+}$ radical (Zhu et al., 2008).

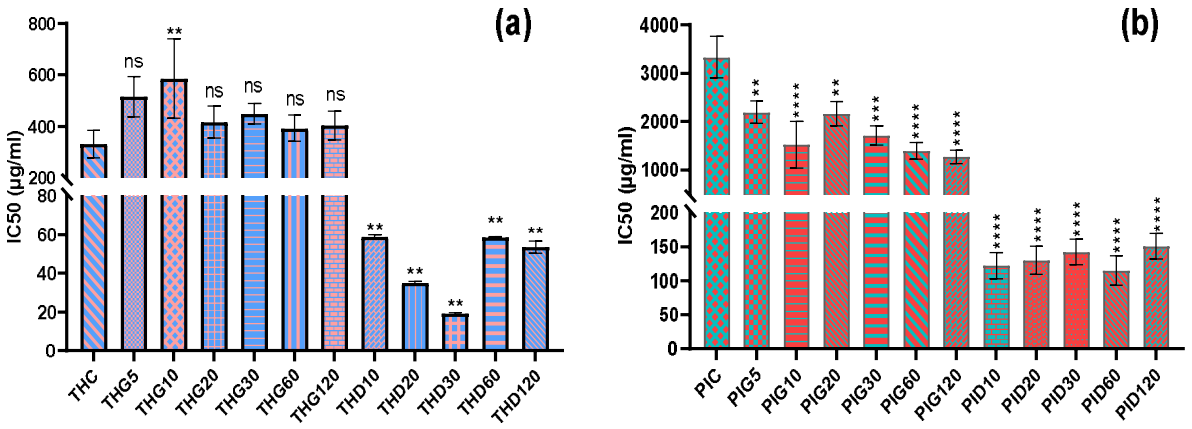


Figure 31: IC_{50} values (µg/mL) of TH digests (a) and PI digests (b) for $ABTS^{\bullet+}$ scavenging activity. Values are expressed as mean ± SD, n = 3. ns = non-significant; **p < 0.01; ***p < 0.001; ****p < 0.0001 compared with the control group (THC and PIC).

III.2.4.3. Fe^{2+} chelation activity

Further investigation was conducted to assess the post-gastrointestinal digestion antioxidant potential of TH and PI, focusing on their Fe^{2+} chelation activity. As depicted in Figure 31.a, the chelating activity of TH exhibited an increase after 2h of gastric digestion involving pepsin (from IC_{50} = 22.191 ± 0.924 to 14.407 ± 1.072 µg/mL), while no significant ($p > 0.05$) variation was observed following a 2-hour gastric digestion of PI (Figure 32.b).

Upon an additional 2-hour intestinal digestion with pancreatin, the IC_{50} of TH experienced a minor rise, reaching an IC_{50} value of 13.161 ± 0.082 µg/mL. Conversely, a substantial increase was noticeable for PI (from IC_{50} = 352.765 µg/mL for PID 120 to 11.841 ± 1.485 µg/mL). This heightened ability of digestates to chelate Fe^{2+} could potentially be attributed to the distinct peptide structures and specific amino acid side chain groups. These components likely play a pivotal role in terminating free radical chain reactions and effectively binding transition metal ions (Decker et al., 1992).

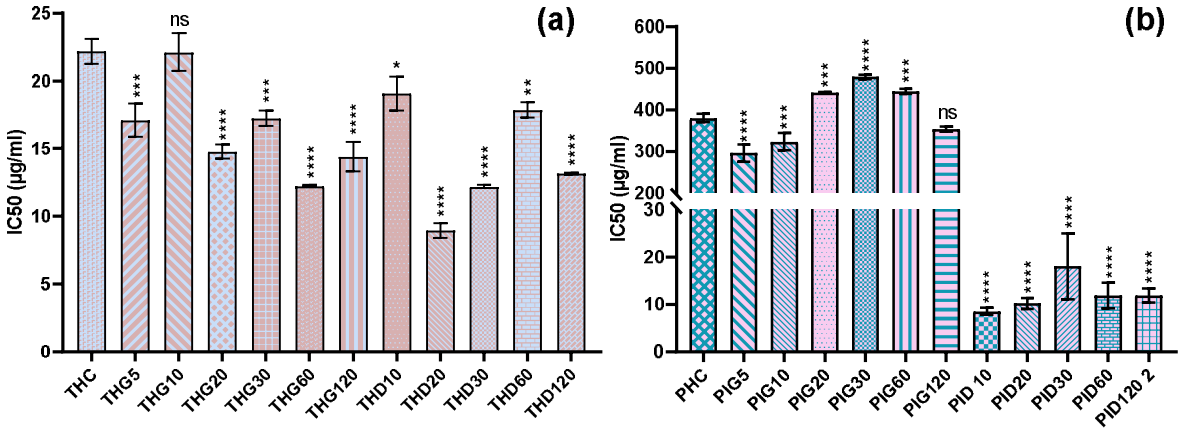


Figure 32: IC_{50} values (µg/mL) of TH digests (a) and PI digests (b) for Fe^{2+} chelating activity. Values are expressed as mean ± SD, n = 3. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 compared with the control group (THC and PIC).

III.2.4.4. FRAP

The capacity for reducing power gauges the potential of a substance to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) by donating electrons or hydrogen (Arise et al., 2016). In this investigation, the iron-reducing capabilities of TH and PI digests were examined at a peptide concentration of 1 mg/mL. Notably, no significant difference (p > 0.05) emerged in the reducing power ability of TH digests. The absorbance at 700 nm for the undigested sample (THC) stood at 0.636 ± 0.002, while those after 2h of gastric digestion and 2h of intestinal digestion were 0.677 ± 0.049 and 0.694 ± 0.019, respectively. This indicates that the reducing power of TH remained unaffected throughout simulated gastrointestinal digestion.

On the other hand, the reducing power propensity of PI exhibited a significant divergence

between gastric phase digests and intestinal phase counterparts. However, no significant difference emerged between the reducing power of undigested PI (PIC) and digests following a 2-hour gastric digestion ($OD_{700\ nm}$ = 0.115 ± 0.027 for PIC and $OD_{700\ nm}$ = 0.111 ± 0.015). Remarkably, the reducing power ability of PI digests underwent a significant increase ($p < 0.0001$) during simulated intestinal digestion, reaching an $OD_{700\ nm}$ of 0.214 ± 0.003.

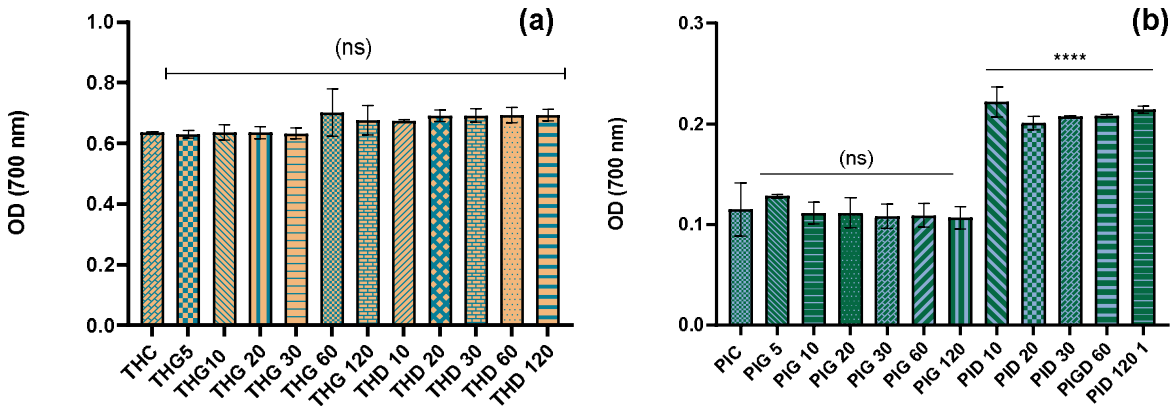


Figure 33: Reducing power assay of TH digests (a) and PI digests (b) measured at 700 nm. Values are expressed as mean ± SD, n = 3. ****p < 0.0001, ns = non-significant compared with the control group (THC and PIC).

III.2.4.5. OH radical scavenging activity

The assessment of OH radical scavenging potential for TH and PI digests is shown in Figure 34. Notably, the OH radical scavenging activity displayed a significant decrease ($p < 0.0001$) during gastric digestion. This transitioned from an IC_{50} value of 53.511 ± 2.656 µg/mL for THC to 215.846 ± 6.94 µg/mL for THG120. However, a notable increase was evident after intestinal digestion, where the final digest yielded an IC_{50} of 99.083 ± 1.757 µg/mL following 4h of gastrointestinal digestion. This result suggests a reduction in OH radical scavenging activity for the trypsin hydrolysate due to simulated gastrointestinal digestion, yet the antioxidant potential remained considerable.

In the case of PI, the control that underwent non-enzymatic treatment exhibited no significant alteration ($p > 0.05$) in activity compared to its digests (PIG120), obtained after 120 min of gastric digestion. However, an observable increase in activity was registered after intestinal

digestion, culminating in the highest activity for PID120 with an IC_{50} value of 79.964 ± 0.958 µg/mL. Intriguingly, the final PI digest post-gastrointestinal digestion displayed superior OH radical scavenging activity compared to the corresponding TH digest. This suggests that the application of digestive enzymes to PI during gastrointestinal digestion released more potent antioxidant amino acids, particularly aromatic amino acids like Phe, Tyr, and Trp, thereby enhancing the antioxidant activity of PI digestates (Aluko and Monu, 2003).

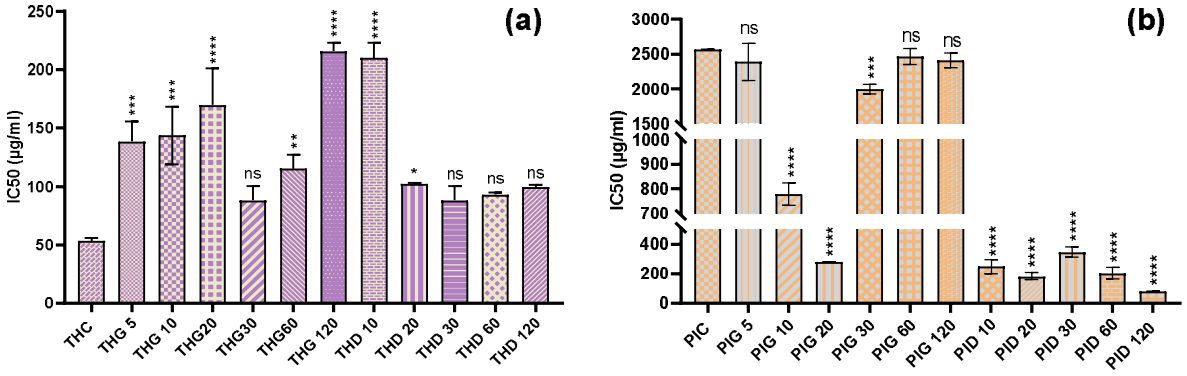


Figure 34: IC_{50} values (µg/mL) of TH digests (a) and PI digests (b) for scavenging activity. Values are expressed as mean ± SD, n = 3. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 compared with the control group (THC and PIC).

III.2.4.6. ORAC assay

The ORAC assay serves to evaluate scavenging capacity through a hydrogen-atom transfer mechanism. This involves exposing the sample to a peroxyl radical generator (AAPH), and measuring the oxidative degradation of fluorescein (Ou et al., 2001). The protective effect of antioxidants is quantified by measuring the area under the fluorescein decay curve (AUC), providing insight into the degree of scavenging. Different Trolox concentrations were tested, and ORAC values were expressed as µmol TE/g protein.

ORAC values demonstrated that gastric digestion led to a reduction in antioxidant activity for both TH and PI (Figure 35), except for PIG3, which exhibited increased antioxidant activity. The diminished activity during gastric digestion could be attributed to certain amino acids with antioxidant properties potentially being concealed within the steric structure, rendering them inactive. In contrast, intestinal digestion amplified the antioxidant activity of both TH and PI

digests, resulting in notable ORAC values of 1227.062 ± 83.934 µmol/g protein and 1374.445 ± 20.886 µmol/g protein, respectively. As the ORAC assay gauges the antioxidant capacity in both inhibiting radical initiation and neutralizing formed radicals (Ma et al., 2010), the heightened antioxidant activity during intestinal digestion suggests the release of amino acids capable of inhibiting radical initiation and neutralizing peroxyl radicals.

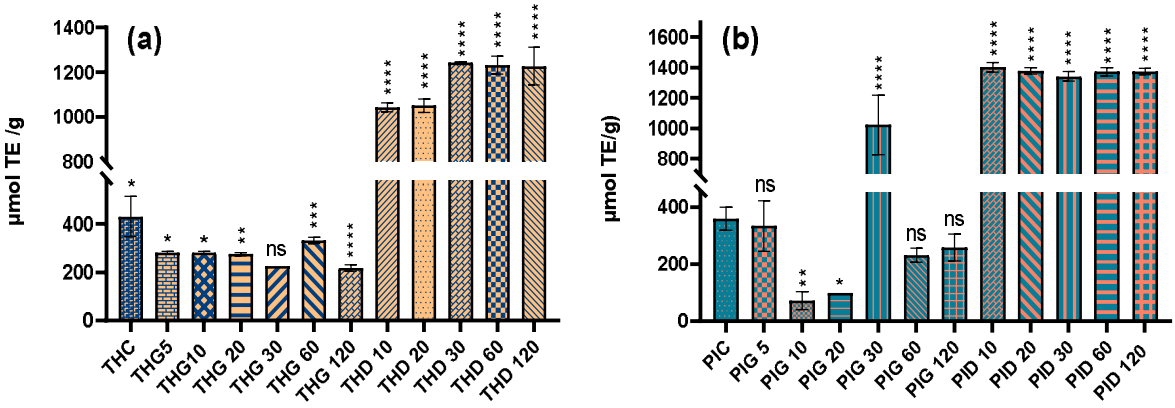


Figure 35: ORAC values of TH digests (a) and PI digests (b) expressed in µmol TE/g protein. Values are expressed as mean ± SD, n = 3. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 compared with the control group (THC and PIC).

Among the i*n vitro* assays utilized to assess sample antioxidant activity, ORAC offers a distinct advantage over FRAP, DPPH, and $ABTS^{\bullet+}$ scavenging assays. This is due to its biological relevance, as ORAC replicates physiological conditions by incorporating peroxyl radicals found in the human body. These radicals result from hydroperoxide homolysis during fatty acid oxidation (Almeida et al., 2008).

III.2.5. *In vitro* assessment of allergenic reactivity

III.2.5.1. Western Blot

A Western Blot analysis was conducted to assess the IgE-binding capability of pine nut proteins using sera from pine nut-allergic patients. The Western blot results using serum from pine nut-allergic patients indicated positive reactivity of their IgE antibodies with protein fragments of PI and PIL having molecular weights of 49, 36, 20, 14, and 11 kDa, along with smaller bands at 25, 27, and 34 kDa. Toomer et al. (2013), previously reported that pine nut proteins with molecular

weights of 150, 113, 84, 50, 36, and 17 kDa displayed immunoreactivity with antibodies from sera of pine nut-allergic patients.

In contrast, the trypsin hydrolysate protein bands (5-10, 11, 14, and 16 kDa) did not exhibit IgE immunoreactivity to the sera of pine nut-allergic individuals. However, minimal, and barely noticeable immunoreactivity was observed for the 20 and 21 kDa protein bands. These findings suggest that trypsin hydrolysis led to a reduction in the IgE-binding capacity of the proteins by disrupting the IgE-binding epitopes present within the allergenic protein fragments. To further verify the reduction in TH allergenicity, a more sensitive technique such as Inhibition Elisa should be employed.

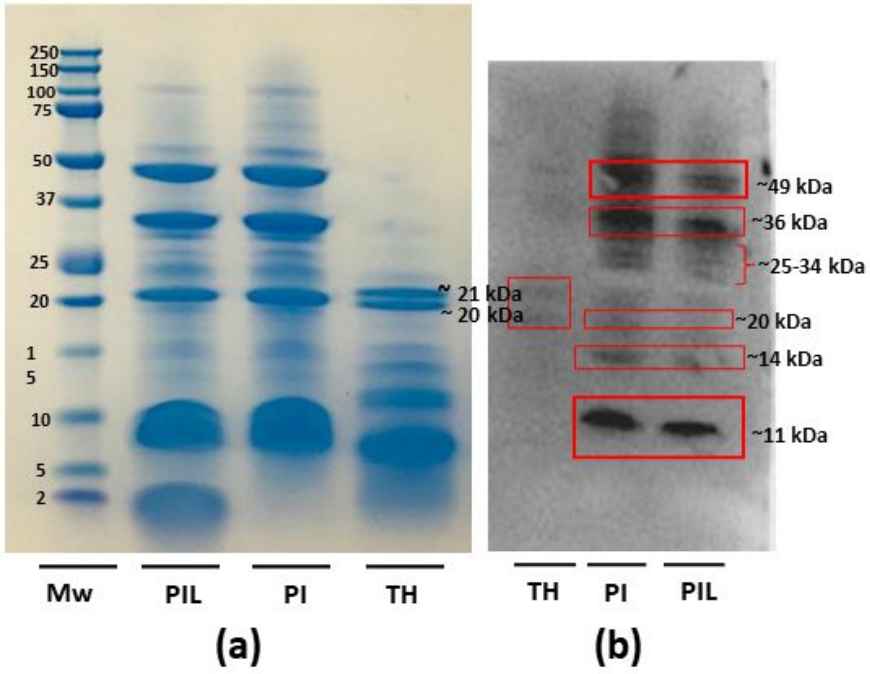


Figure 36: SDS-PAGE (a) and IgE-western Blot (b) of TH, PI, and PIL.

III.2.5.2. Elisa Inhibition

Unlike proteins, small peptides exhibit less immobilization in immunoblots. For further investigation, it is advisable to employ ELISA inhibition, a technique renowned for its heightened sensitivity in detecting IgE-reactive proteolytic fragments. ELISA inhibition was employed for TH and using PI as an antigen, as depicted in Figure 37. The results indicate that the protein isolate demonstrated a 100 % capacity to bind IgE from pine nut allergic patients, whereas TH exhibited a mere 0.057 % binding ability, thereby corroborating the results of the western Blot analysis. The

application of trypsin-induced hydrolysis has effectively diminished the allergenic potential of pine nut proteins by disrupting the IgE-binding epitopes inherent in the allergens.

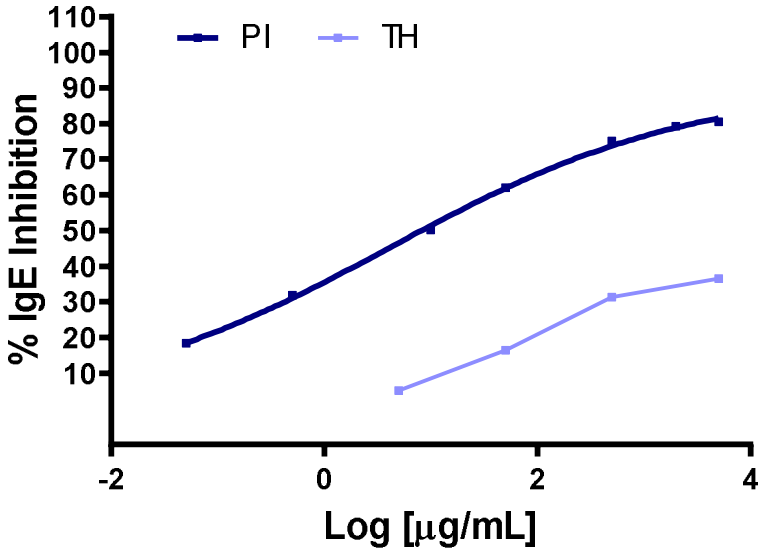


Figure 37: The percentage of IgE inhibition of TH and PI (inhibitor).

A previous study conducted by Crespo et al. (2021) explored the IgE-binding epitopes of the allergen Pin p1 using sera from patients with well-characterized clinical allergy to pine nuts. Their analysis revealed three regions of Pin p1 containing five epitopes recognized by patient sera. Furthermore, they highlighted that these epitopes were exposed on the surface, facilitating easy access for IgE binding to the highly resistant structure of Pin p1. The constructed 3-dimensional structure of Pin p1 indicated that the IgE-binding epitopes were α-helices and coils.

III.3. Conclusion

TH, whose potent antioxidant activity was demonstrated in the previous study, was subjected to simulated gastrointestinal digestion to assess its effect on the antioxidant activity of TH. Results revealed that gastrointestinal digestion didn't affect the FRAP of TH; Furthermore, it increased the ABTS radical scavenging activity, Fe^{2+} chelating activity, and ORAC values of TH. However, it led to a decrease in its the DPPH radical scavenging activity and OH radical scavenging activity, though the activity was still considered as good. The allergenicity of PI and TH was tested by assessing their immunoreactivity to IgE antibodies present in the sera of pine nut-allergic patients. Results demonstrated a reduction in the immunoreactivity for TH compared

to PI. In conclusion, TH's antioxidant activity persisted even after undergoing simulated gastrointestinal digestion, highlighting its ability to function as antioxidants within the body. The reduced allergenicity showcased that enzymatic hydrolysis using trypsin is an effective process to reduce the allergenicity of proteins.

General conclusion and perspectives

General conclusion and perspectives

The need to find alternatives to animal-derived proteins stems from concerns about the environmental impact and resource inefficiency of traditional animal protein production, as well as the potential for improved health outcomes through plant-based options. Diversifying protein sources can enhance food security, take account of cultural and dietary diversity, and harness innovation and technology to create economically viable alternatives. In this process, these initiatives collectively address the challenges of sustainability, nutrition, ethics, and social well-being.

Throughout the course of this thesis, accomplishing the major objectives has enabled us to highlight several outcomes. The initial objective, focused on " Optimization of alkali extraction conditions of *Pinus pinea* L. nuts protein isolates, evaluation of their functional properties and biochemical characterization, " revealed the impact of extraction pH, extraction time, and solvent-to-sample ratio on the purity, solubility, and emulsifying activity of protein isolates. The study demonstrated that extraction pH significantly was a highly influential factor, with a pH range of 8 to 8.86 yielding protein isolates with high solubility (102.1%), strong emulsifying activity (33.22 m^2/g), and high purity (98.76 g/100 g protein). The optimal extraction conditions suggested by the model and validated were pH 8, time of 108 minutes, and a solvent-to-sample ratio of 1:10 g/mL. Furthermore, the assessment of functional properties of the optimized *Pinus pinea* L. nuts protein isolate (OPPPI), in comparison with commercial soy protein isolate (SPI) and pea protein isolate (PPI), demonstrated that OPPPI exhibited superior solubility, oil holding capacity, foaming and emulsion stabilities compared to SPI and PPI. Although its emulsifying activity was similar, OPPPI displayed lower water holding capacity compared to SPI and a higher foaming capacity than PPI but lower than SPI. Additionally, a detailed biochemical characterization of OPPPI unveiled its predominant composition of 7S globulin, 11S globulin, and 2S albumin, with β-sheet as its primary secondary structure. Ultimately, the favorable functional properties of OPPPI underscore its potential as a promising ingredient in the food industry, offering diverse applications as a plant-based protein alternative in various food products.

The second objective of this study, pursued in " Antioxidant activity of *Pinus pinea* L. nuts protein hydrolysates produced using different enzymes," illustrated that employing distinct enzymes—pepsin, trypsin, and Alcalase—for enzymatic hydrolysis, both individually and sequentially, resulted in the generation of protein hydrolysates with unique attributes in terms of

their degree of hydrolysis, peptide composition, and antioxidant efficacy. All the protein hydrolysates exhibited notable antioxidant properties. Notably, trypsin hydrolysate (TH) exhibited the most significant activity in terms of DPPH radical scavenging, Fe^{2+} chelation, and OH radical scavenging activities. Alcalase hydrolysate (AH) displayed the highest ORAC value, pepsin hydrolysate (PH) exhibited superior FRAP, and the pepsin/Alcalase hydrolysate (P/A H) demonstrated the highest $ABTS^{\bullet+}$ radical scavenging activity. The variability in antioxidant activity among the hydrolysates can be attributed to the differences in their peptide sequences. High-performance liquid chromatography-mass spectrometry (HPLC-MS/MS) analysis of TH, P/T H, and T/A H confirmed the presence of distinct peptide sequences with varying amino acid compositions. The antioxidant activity of these hydrolysates could be linked to the presence of specific amino acids, such as Alanine, Leucine, Valine, Proline, Phenylalanine, and Tyrosine, which have been recognized for their contribution to the antioxidant properties of peptides.

Finally, the ultimate objectives of this thesis, explored in "Immunoreactivity of pine nuts (*Pinus pinea* L.) protein isolate and trypsin hydrolysate and the effects of simulated *in vitro* gastrointestinal digestion on the antioxidant activity of trypsin hydrolysate," showcased that subjecting the trypsin hydrolysate to simulated *in vitro* gastrointestinal digestion did not diminish its antioxidant efficacy when evaluated through $ABTS^{\bullet+}$ radical scavenging, Fe^{2+} chelation, and ORAC assays. Despite digestion, its FRAP activity remained unchanged; however, the OH radical scavenging and DPPH radical scavenging activities experienced reduction, albeit still retaining considerable activity levels. Furthermore, analyzing the SDS-PAGE profile of the trypsin hydrolysate at different points during gastrointestinal digestion allowed us to monitor the progression of the digestion across the dual phases. At the conclusion of gastrointestinal digestion, it was established that only di- and tripeptides along with free amino acids remained. The results underscore the release of antioxidant peptides from TH following gastrointestinal digestion, pointing to their potential for exerting antioxidant effects *in vivo* due to their bioavailability. In another aspect, the allergic potential of the protein isolate and trypsin hydrolysate was evaluated using sera from pine nuts-allergic patients. The results indicated that trypsin hydrolysis contributed to a reduction in pine nut allergy (% IgE-binding = 0.057%), further confirming the safety of trypsin hydrolysate.

The findings presented within this thesis validate the hypotheses initially proposed. The investigations undertaken throughout this study have provided us with novel insights into the

protein isolates and protein hydrolysates derived from *Pinus pinea* L. nuts. These insights encompass the functional attributes of protein isolate, antioxidant potential and bioavailability of protein hydrolysates, along with the allergenic characteristics of both protein isolate and trypsin hydrolysate. In summary, pine nuts (*Pinus pinea* L.) hold great promise as a valuable reservoir of protein components, whether deployed as functional proteins or as nutraceuticals boasting commendable functional properties and antioxidant capabilities.

Building upon these promising initial findings, which underscore the potential of our approach, several avenues warrant recommendations to broaden the scope for generating protein isolates and peptides of interest, optimizing their application. The following proposals are some examples:

- A scale-up at an industrial level is imperative to explore the behavior of protein isolates within food matrices, optimizing control and conservation strategies for protein isolate, and assessing the economic viability of their integration into the food industry.
- In the context of trypsin hydrolysate, it holds potential to pursue further isolation and characterization of individual peptide sequences. This would serve the purpose of identifying novel peptide sequences and validating their bioactivities, especially in terms of antioxidant properties, while also examining other biological activities.
- An essential step concerning the identified bioactive sequences is to delve into an in-depth study of their mechanisms of action.
- An *in vivo* investigation of bioactive peptides is indispensable to validate the observed effects *in vitro*. An intriguing avenue would involve testing a mixture of peptides exhibiting the most promising effects across the array of bioactivities assessed *in vitro*. This would serve to verify the positive effects on various anomalies linked to oxidative stress development.
- With regard to the allergenicity of trypsin hydrolysate, a more comprehensive range of *in vitro* and *in vivo* experiments is necessary.

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Appendices

Appendix 1: Calibration curves of BSA, glucose, and L-leucine for the determination of the protein content, carbohydrate content, and degree of hydrolysis, respectively.

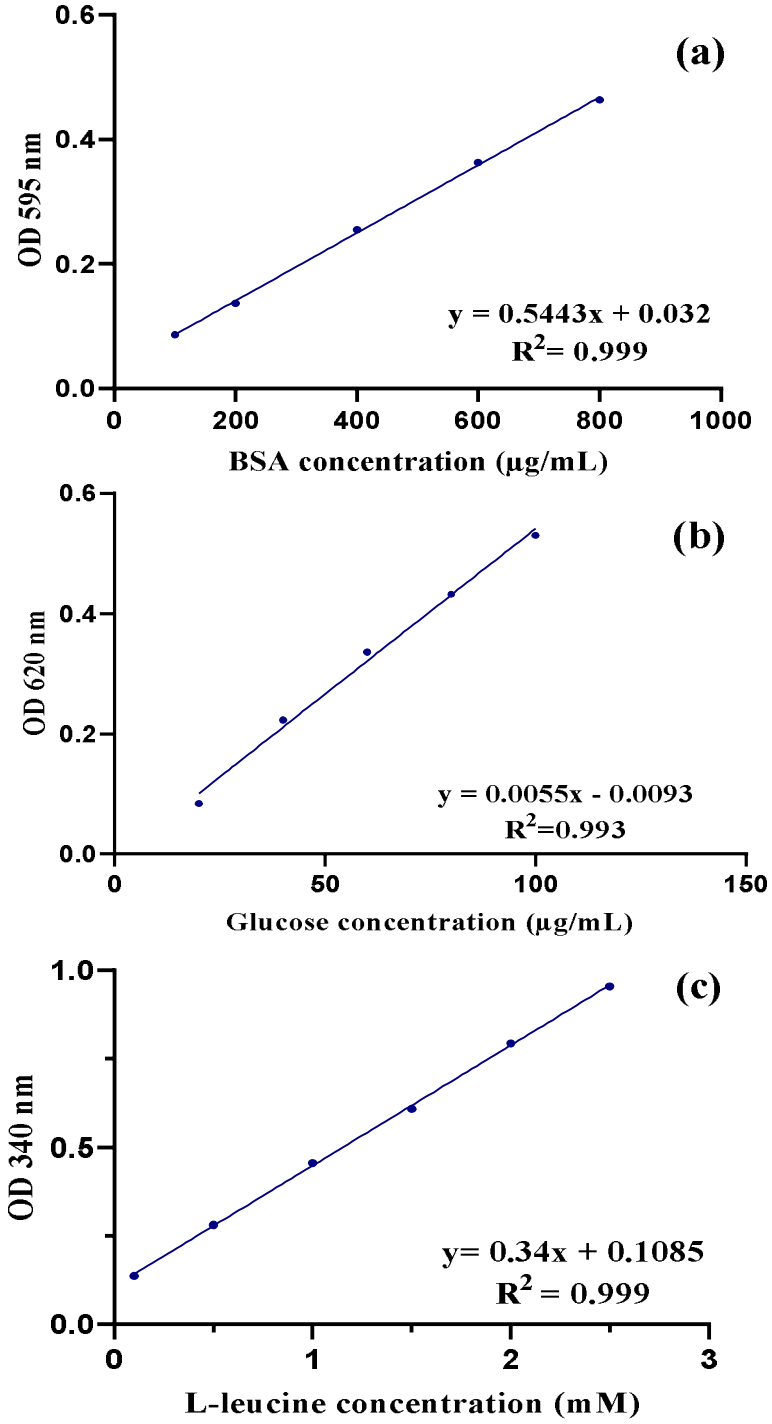

Figure 38: Calibration curves used to measure protein content (a), carbohydrate content (b), and degree of hydrolysis (c).

Appendix 2: Preparation of simulated digestion fluids electrolyte stock solutions (1.25 ×), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF).

Table XVII: Preparation of SGF and SIF stock solutions, volumes are calculated to achieve a final volume of 500 mL for each solution.

Constituents	Molarity (M)	Volume (mL)	
		SGF (pH 3)	SIF (pH 7)
KCl	0.5	6.9	6.8
KH_2PO_4	0.5	0.9	0.8
$NaHCO_3$	1	12.5	42.5
NaCl	2	11.8	9.6
$MgCl_2$	0.15	0.4	1.1
$(NH_4)_2CO_3$	0.5	0.5	/
NaOH	1	/	/
$CaCl_2$	0.3	/	It is added the moment of digestion
HCl	6	Until pH is adjusted to pH 3	Until pH is adjusted to pH 7
H_2O	/	Until the total volume reaches 500 mL	

Appendix 3: Antioxidant activity of trypsin hydrolysate and protein isolate measured by DPPH and ABTS radicals scavenging activity, OH radical scavenging activity, and Fe^{2+} chelating activity.

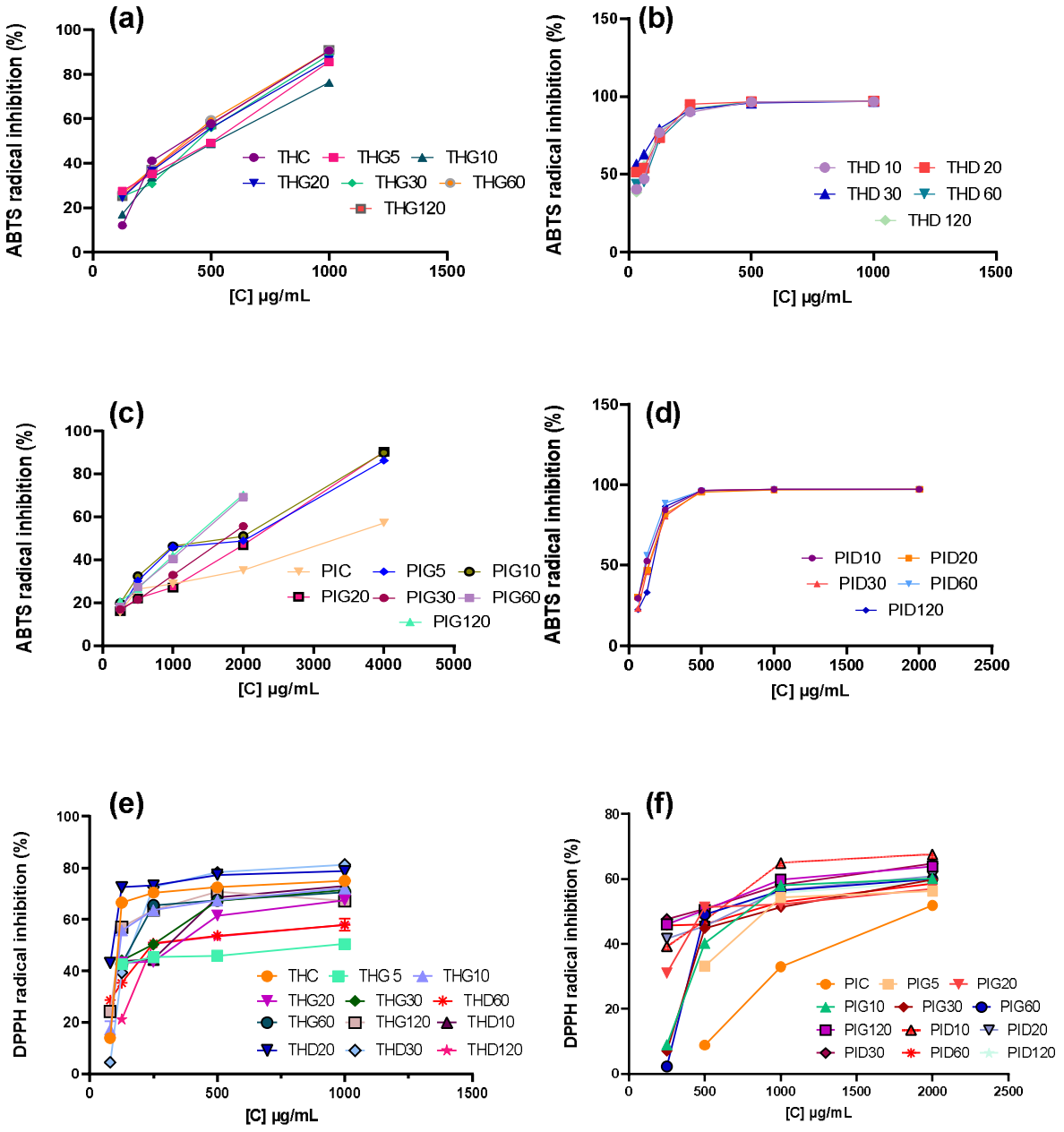

Figure 39: Antioxidant activity of trypsin hydrolysate and protein isolate measured by DPPH radical scavenging activity (e-f), and ABTS radical scavenging activity (a-d).

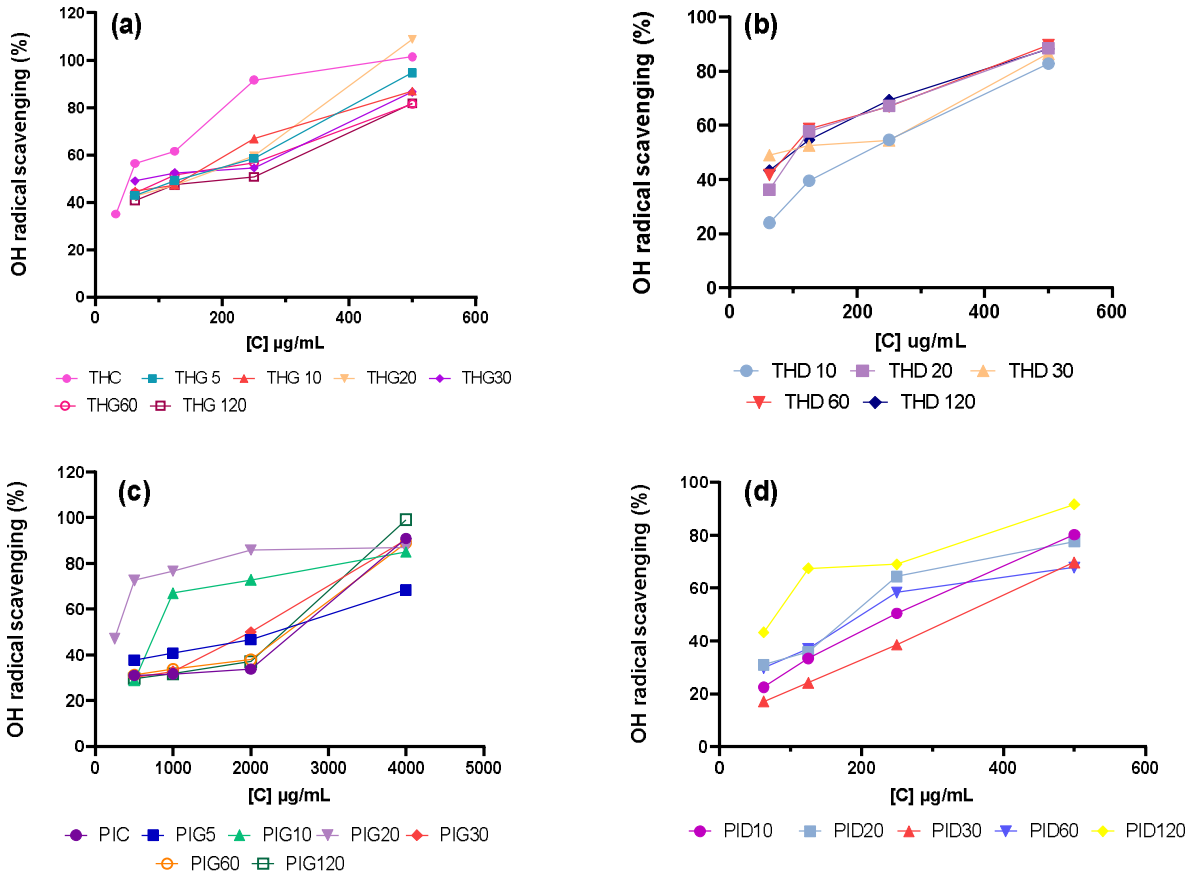

Figure 40: Antioxidant activity of trypsin hydrolysate (a) and protein isolate (b-c) measured by OH radical scavenging activity.

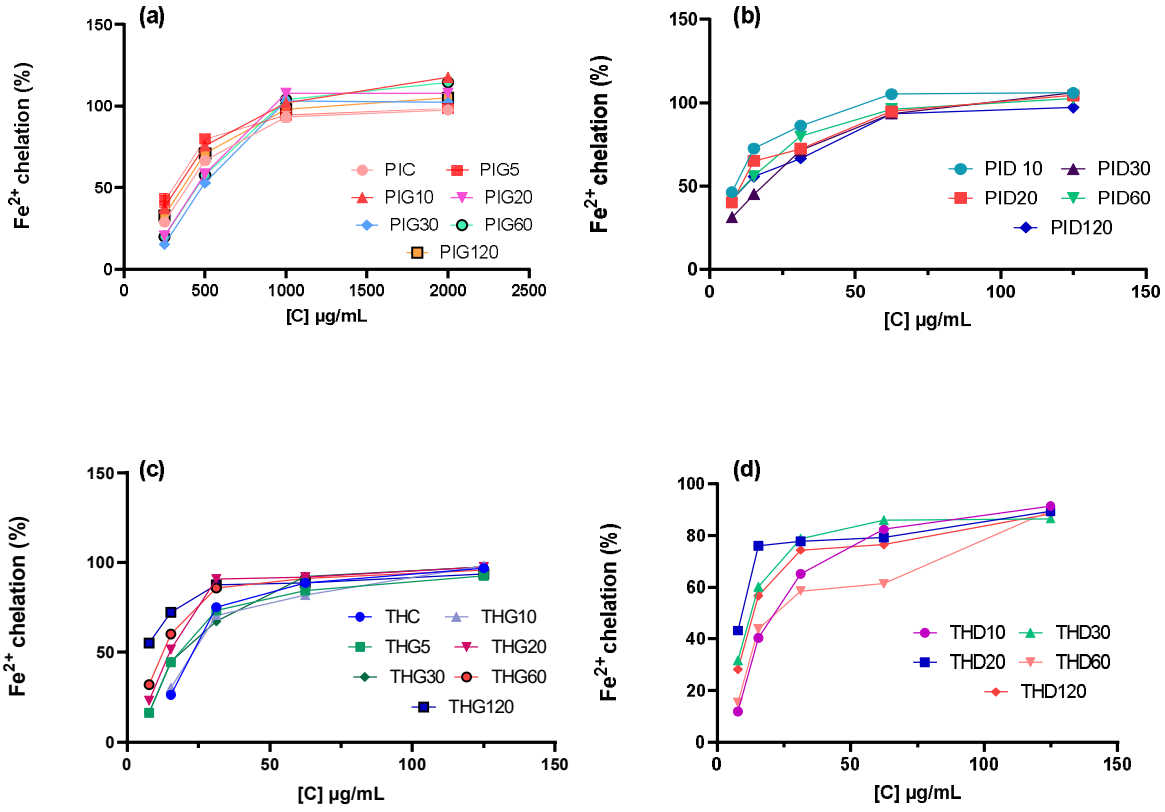

Figure 41: Antioxidant activity of trypsin hydrolysate (a-b) and protein isolate (c-d) measured by Fe^{2+} chelating activity.

Scientific publications and communications

Scientific publications and communications

➢ Scientific publications

1. Pine nuts (*Pinus pinea* L.) as a potential novel plant-based source of functional protein isolates: Optimization of alkali extraction conditions, evaluation of functional properties, and biochemical characterization. **Benzitoune Nourelimane**, Kadri Nabil, Adouane Meriem, Berkani Farida, Abbou Amina, Dahmoune Farid, Remini Hocine, Bensmail Souhila., (2022). *Journal of Food Processing and Preservation*, *46*(4), e16471. (Pine nuts (Pinus pinea L.) as a potential novel plant-based source of functional protein isolates: Optimization of alkali extraction conditions, evaluation of functional properties, and biochemical characterization - Benzitoune - 2022 - Journal of Food Processing and Preservation - Wiley Online Library).

➢ International communications

1. Evaluation of hypocholesterolemic activity of phenolic extract of an oilseed in high cholesterol diet fed *Wistar albinos* rats. First international symposium, environment and sustainable development, 10-11 February 2020, Relizane (Algeria): Poster. **Nourelimane-elhouda Benzitoune**, Nabil Kadri, Amina Abbou, Meriem Adouane.

2. Evaluation de l'activité hypocholestérolémiante d'un extrait polyphénolique d'une graine oléagineuse *in vivo***.** Third international symposium, Medicinal plants and materials, 25-27 February 2020, Tebessa (Algeria): Poster. **Benzitoune Nourelimane-elhouda**, Kadri Nabil, Abbou Amina, Adouane Meriem.

3. Evaluation of hypocholesterolemic activity of phenolic extract of an oilseed in high cholesterol diet fed *Wistar albinos* rats. Sixth MGIBR International Workshop of Aromatic, Medicinal and condiment plants Virtues and development prospects, 21-22 December 2020, Tlemcen University (Algeria): Poster. **Nourelimane-elhouda Benzitoune**, Nabil Kadri, Amina Abbou, Meriem Adouane.

4. Optimization of alkali extraction conditions of *Pinus pinea* L. nuts protein isolates, evaluation of functional properties and biochemical characterization. International Seminar on Bioresources and Sustainable Development (BDD2023), 3-4 May 2023 Bouira, Alegria: Poster. **Benzitoune Nourelimane-elhouda**, Kadri Nabil, Amina Abbou, Meriem Adouane, Farida BerkanI, Farid Dahmoune, Hocine Remini, Souhila Bensmail.

- **National communications**

1. Evaluation of hypocholesterolemic activity of crude polyphenolic extract of Mediterranean oilseeds in high cholesterol diet fed *Wistar albinos* rats. Séminaire National de Ressources végétales, Produits Naturels et Santé, 9-10-11 Juin 2021, Blida (Algérie): Poster. **BENZITOUNE Nourelimane-elhouda,** KADRI Nabil.
2. Evaluation of hypocholesterolemic activity of crude polyphenolic extract of Mediterranean oilseeds in high cholesterol diet fed *Wistar albinos* rats. Séminaire National de Bio-ressources : Nutrition, Santé et Environnement, 17-18 Mai, 2021, M'sila (Algérie) : Poster. **BENZITOUNE Nourelimane-elhouda,** KADRI Nabil.
3. Potential use of Pinus seeds polyphenols as therapeutic agents: evaluation of *in vivo* anti-inflammatory activity and *in vitro* antioxidant activity. 20 Mai 2021, Mila (Algérie): Poster. **BENZITOUNE Nourelimane-elhouda,** KADRI Nabil.

Abstract

The growing preference for plant-based protein alternatives driven by health consciousness, environmental sustainability, and a demand for eco-friendly options has prompted significant interest in exploring novel sources. This study investigates *Pinus pinea* L. nuts as a case study due to their high protein content and associated health benefits. Pine nuts protein isolates were produced using alkali extraction and isoelectric precipitation method, with the impact of alkali extraction conditions on purity, solubility, and emulsifying activity was investigated using response surface methodology. Subsequent evaluations were carried out to assess the functional properties of the isolates, as well as an analysis of their protein composition by SDS-PAGE, and an assessment of their secondary structure by FTIR spectroscopy.

Additionally, protein hydrolysates were generated from the protein isolate via enzymatic hydrolysis, employing pepsin, trypsin, and Alcalase, both individually and sequentially. Their antioxidant activity was evaluated through various assays, including radical scavenging ($ABTS^{\bullet+}$, DPPH, •OH), iron chelation, ferric reducing antioxidant power, and oxygen radical absorbance capacity, revealing notable outcomes with trypsin hydrolysis. Through in-depth analysis using high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS), distinctive peptide sequences were identified within the protein hydrolysates.

To assess bioavailability, simulated *in vitro* gastrointestinal digestion was performed on trypsin hydrolysate and protein isolate. Furthermore, the allergenicity characteristics of trypsin hydrolysate and protein isolate were investigated using IgE-inhibition ELISA and Western Blot techniques with sera from pine nut-allergic individuals. The results indicated that trypsin hydrolysis effectively reduced the allergenicity of pine nuts.

In conclusion, the study highlights the potential of *Pinus pinea* L. nuts as a promising source for valuable protein ingredients. These ingredients can serve as functional proteins in the food industry or as nutraceuticals with antioxidant properties.

Keywords: *Pinus pinea* L. nuts, Alkali extraction, Protein isolates, functional properties, Protein hydrolysates, Antioxidant activity, Simulated *in vitro* gastrointestinal digestion, SDS-PAGE, HPLC-MS/MS, immunoreactivity.

Résumé

La préférence croissante pour les protéines d'origine végétale, motivée par la prise de conscience des problèmes de santé, la durabilité environnementale et la demande d'options respectueuses de l'environnement, a suscité un intérêt considérable pour l'exploration de nouvelles sources. Cette étude porte sur les noix de *Pinus pinea* L. en raison de leur teneur élevée en protéines et des bienfaits pour la santé qui y sont associés. L'impact des conditions d'extraction alcaline sur la pureté, la solubilité et l'activité émulsifiante a été étudié à l'aide de la méthodologie de la surface de réponse. Des évaluations ultérieures ont été effectuées pour évaluer les propriétés fonctionnelles des isolats, ainsi qu'une analyse de leur composition protéique par SDS-PAGE et une évaluation de leur structure secondaire par spectroscopie FTIR.

En outre, des hydrolysats de protéines ont été générés à partir des isolats de protéines par hydrolyse enzymatique, en utilisant la pepsine, la trypsine et l'Alcalase, à la fois individuellement et séquentiellement. Leur activité antioxydante a été évaluée au moyen de différents tests, notamment la capture des radicaux ($ABTS^{\bullet+}$, DPPH, •OH), la chélation du fer, le pouvoir antioxydant réducteur ferrique et la capacité d'absorption des radicaux d'oxygène, révélant des résultats notables avec l'hydrolyse à la trypsine. Grâce à une analyse approfondie utilisant la chromatographie liquide haute performance avec spectrométrie de masse en tandem (HPLC-MS/MS), des séquences peptidiques distinctives ont été identifiées dans les hydrolysats de protéines.

Pour évaluer la biodisponibilité, une digestion gastro-intestinale simulée *in vitro* a été effectuée sur l'hydrolysat de trypsine et l'isolat de protéine. En outre, les caractéristiques d'allergénicité de l'hydrolysat de trypsine et de l'isolat de protéine ont été étudiées à l'aide de techniques ELISA d'inhibition des IgE et Western Blot avec des sérums d'individus allergiques aux pignons de pin. Les résultats indiquent que l'hydrolyse de la trypsine réduit efficacement l'allergénicité des pignons de pin.

En conclusion, cette étude met en évidence le potentiel des pignons de *Pinus pinea* L. en tant que source prometteuse d'ingrédients protéiques de valeur. Ces ingrédients peuvent servir de protéines fonctionnelles dans l'industrie alimentaire ou de nutraceutiques aux propriétés antioxydantes.

Mots-clés : les noix de *Pinus pinea* L., extraction alcaline, isolats de protéines, propriétés fonctionnelles, hydrolysats de protéines, activité antioxydante, digestion gastro-intestinale simulée *in vitro*, SDS-PAGE, HPLC-MS/MS, immunoréactivité.

ملخص

إنّ التفضيل المتزايد لبدائل البروتين النباتي مدفوعًا بالوعي الصحي و الإستدامة البيئية و الطلب على الخيارات الصديقة للبيئة قد أثار اهتمامًا كبيرًا باستكشاف مصادر جديدة. تتناول هذه الدراسة الصنوبر الثمري (*Pinus pinea* L.) كدراسة حالة بسبب محتواها العالي من البروتين و الفوائد الصحية المرتبطة بها. تم إنتاج عزلات بروتين الصنوبر باستخدام طريقة الإستخلاص القلوي و الترسيب الكهروضوئي، مع دراسة تأثير ظروف الإستخلاص القلوي على النقاوة و نشاط الإستحلاب باستخدام منهجية سطح الإستجابة. تمّ إجراء التقييمات اللاحقة لتقييم الخصائص الوظيفية للعزلات، بالإضافة إلى تحليل تركيب البروتين الخاص بها بواسطة SDS-PAGE ، وتقييم بنيتها الثانوية بواسطة التحليل الطيفي FTIR.

بالإضافة إلى ذلك، تمّ إنتاج هيدروليزات البروتين من البروتين المعزول عن طريق التحلل المائي الأنزيمي، باستخدام البيبسين والتربسين والألكالاز، بشكل فردي ومتتابع. تمّ تقييم نشاطها المضاد للأكسدة من خلال فحوصات مختلفة، بما في ذلك الكسح الجذري (DPPH,•OH، $ABTS^{\bullet+}$)، وإزالة معدن ثقيل من الحديد، والحد من قوة مضادات الأكسدة، وقدرة امتصاص جذري الأكسجين. ممّا يكشف عن نتائج ملحوظة مع التحلل المائي للتربسين. من خلال التحليل المتعمق باستخدام التحليل اللوني السائل عالي الأداء ومطياف الكتلة الترادفي (HPLC-MS/MS)، تم تحديد تسلسلات الببتيد المميزة داخل هيدروليزات البروتين.

لتقييم التوافر البيولوجي، تم إجراء محاكاة الهضم الهضمي في المختبر على هيدروليزات التربسين وعزل البروتين. بالإضافة إلى ذلك، تمّ دراسة خصائص الحساسية لهيدروليزات التربسين وعزل البروتين باستخدام تقنيات ELISA لتثبيط IgE وتقنيات اللطخة الغربية مع الأمصال المأخوذة من الأفراد الذين يعانون من حساسية الصنوبر. أشارت النتائج إلى أن التحلل المائي للتربسين يقلل بشكل فعال من حساسية الصنوبر الثمري.

في الختام، تؤكد الدراسة على إمكانات الصنوبر الثمري كمصدر واعد لمكونات البروتين القيّمة. يمكن أن تكون هذه المكونات بمثابة بروتينات وظيفية في صناعة الأغذية أو كمواد مغذية ذات خصائص مضادة للأكسدة.

الكلمات المفتاحية: جوز .*Pinus pinea* L، الاستخلاص القلوي، عزلات البروتين، الخواص الوظيفية، هيدروليزات البروتين، نشاط مضادات الأكسدة، محاكاة الهضم الهضمي في المختبر، SDS-PAGE ، HPLC-MS/MS، النشاط المناعي.