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Review

Protein Hydrolysate from Underutilized Legumes: Unleashing the Potential for Future Functional Foods

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ABSTRACT: Proteins play a vital role in human development, growth, and overall health. Traditionally, animal-derived proteins were considered the primary source of dietary protein. However, in recent years, there has been a remarkable shift in dietary consumption patterns, with a growing preference for plant-based protein sources. This shift has resulted in a significant increase in the production of plant proteins in the food sector. Consequently, there has been a surge in research exploring various plant sources, particularly wild, and underutilized legumes such as *Canavalia*, *Psophocarpus*, *Cajanus*, *Lablab*, *Phaseolus*, and *Vigna*, due to their exceptional nutraceutical value. This review presents the latest insights into innovative approaches used to extract proteins from underutilized legumes. Furthermore, it highlights the purification of protein hydrolysate using Fast Protein Liquid Chromatography. This review also covers the characterization of purified peptides, including their molecular weight, amino acid composition, and the creation of three-dimensional models based on amino acid sequences. The potential of underutilized legume protein hydrolysates as functional ingredients in the food industry is a key focus of this review. By incorporating these protein sources into food production, we can foster sustainable and healthy practices while minimizing environmental impact. The investigation of underutilized legumes offers exciting possibilities for future research and development in this area, further enhancing the utilization of plant-based protein sources.

Keywords: characterization, extraction techniques, protein hydrolysate, purification, underutilized legumes

INTRODUCTION

The current trend in healthy eating has significantly influenced the food industry, leading to the development of healthy foods that promote overall well-being. According to the International Food Information Council (2021), a majority of consumers now prioritize meals they perceive as healthier and more environmentally friendly. In a study conducted by Miller et al. (2022), the average global consumption of processed red meat and unprocessed red meat per person in 2018 was 51 g/d and 17 g/d, respectively. Considering the high cost and limited availability of animal proteins, substituting them with plant-based alternatives like legumes can be an environmentally favorable solution (Kristensen et al., 2016).

Plant-based diets are gaining popularity in western nations, and the adoption of vegetarian or vegan lifestyles is an emerging trend (Alcorta et al., 2021). The term "plant-based" encompasses a wide range of foods that primarily originate from plants such as legumes, nuts, fruits, whole grains, vegetables, and oils. However, it can also encompass a small amount of animal-origin foods, including fish, milk, meat, and eggs (Fehér et al., 2020). Individuals following a plant-based diet are not permanently restricted from consuming animal products but have the option to replace them with vegetable-based alternatives.

The ongoing debate regarding the ability of vegetarian diets to meet protein requirements has been a subject of discussion, as these diets exclude animal meat and often limit or eliminate other animal-derived products, which are rich sources of protein (Mariotti and Gardner, 2019). Studies have shown that plant-based protein sources generally have lower levels of certain amino acids, such as threonine, tryptophan, lysine, and methionine (Chardigny and Walrand, 2016). However, a well-planned and balanced diet that primarily consists of plant-based foods can effectively provide all the necessary amino acids, as they can be combined and complemented to prevent pro-

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tein deficiencies (Chardigny and Walrand, 2016).

Protein hydrolysate production is currently a prominent area of research due to its economic viability and potential to address environmental concerns (Harnedy and FitzGerald, 2012). Literature suggests that legume protein hydrolysates play a crucial role in extracting bioactive peptides, which have the potential to prevent and treat various illnesses such as cancer, immunological disorders, infections, and cardiovascular disease (Kamran and Reddy, 2018). The presence of bioactive peptides in legumes and cereals offers an opportunity to enhance the quality and functionality of commonly consumed foods, thereby improving overall nutritional value (Malaguti et al., 2014).

Promoting legume consumption and developing affordable products for underprivileged populations play a vital role in poverty reduction and addressing malnutrition. In underdeveloped African and Asian countries, approximately 170 million preschoolers and nursing mothers suffer from protein-energy malnutrition, a severe nutritional condition (Nedumaran et al., 2015). In response to this challenge, researchers worldwide are focusing their efforts on utilizing naturally occurring, wild, and underutilized legumes that have been overlooked, neglected, or are region-specific (Bhat and Karim, 2009). In light of this, this review emphasizes the potential of exploring underutilized legumes as a valuable source for future functional foods.

PROTEIN

Proteins, macromolecules composed of polymers called amino acids, serve diverse functions in the body (Blanco and Blanco, 2017). They act as catalysts, transport and store molecules like oxygen, provide immune defense, and mechanical support, control activity, transmit nerve impulses, and regulate growth and differentiation (Berg et al., 2002). Protein molecules can be characterized by up to four structural levels (Horton et al., 2002). The term "primary structure" originally referred to a protein's complete covalent structure, but it is now commonly used to describe the arrangement of amino acids in each polypeptide chain that constitutes the protein (Breda et al., 2008). The secondary structure refers to regular local conformations stabilized by hydrogen bonds between carbonyl oxygens and amide hydrogens of the peptide backbone, with α-helices and β-strands being the main types of secondary structures (Horton et al., 2002). The specific arrangement of amino acids in the peptide chains determines whether they fold into helices, from pleated sheets, or adopt other structures (Litwack, 2018). The compact and folded polypeptide chain is referred to as the tertiary structure while the assembly of two or more polypeptide chains forms the quaternary structure of an oligomeric or multisubunit protein (Horton et al., 2002).

Generally, animal-derived proteins have amino acid sequences that closely resemble those found in the human body, containing all nine essential amino acids in appropriate proportions (FAO, 2013). Animal proteins are known for their high digestibility, net utilization, biological value, and protein digestibility-corrected amino acid score (Ismail et al., 2020). However, animal proteins are often expensive and not easily accessible, leading to a growing interest in substituting them with vegetable-based protein sources, such as legumes (Kristensen et al., 2016). Consequently, there has been a global increase in research focused on exploring vegetable protein sources, particularly legumes (Chel-Guerrero et al., 2002).

Plant protein is now recognized as a healthy option to meet protein requirements and recommendations, despite its previous reputation as a less nutritious and incomplete source of essential amino acids (Richter et al., 2015). By considering the amino acid deficiencies of individual plant-based meals, combining the right plant protein sources can enhance the overall protein quality of a complete meal. However, the production of conventional legumes like soybean falls short of meeting the needs of a growing population and the animal feed industry (Chaturvedi et al., 2015). Consequently, there is a need to explore and utilize the underutilized legumes to address this demand.

NUTRITIONAL VALUE OF UNDERUTILIZED LEGUMES

Plants belonging to the Leguminosae family, commonly known as Fabaceae, are categorized as legumes due to their ability to produce seeds within pods (Kouris-Blazos and Belski, 2016). In recent years, pulses have been recognized as excellent sources of plant protein, challenging the perception that they were inferior to meat. Legumes offer significant nutritional value by providing essential vitamins, minerals, unsaturated fats, complex carbohydrates, dietary fiber, and proteins containing essential amino acids contributing to a well-rounded human diet (Maphosa and Jideani, 2017). Extensive research has been conducted to explore the potential benefits of plant proteins in reducing the prevalence of cancer, diabetes, and cardiovascular diseases (Sharma and Thakur, 2022). Consequently, plant-based proteins are being extensively investigated for their potential to serve as beneficial food options (Hertzler et al., 2020).

Consequently, there has been a global increase in research focusing on vegetable protein sources, particularly legumes (Zainol et al., 2020). However, the production of conventional legumes falls short in meeting the demands of a growing population and the animal feed industry

(Chaturvedi et al., 2015). As a result, there is a growing need to explore and utilize underutilized legumes. Moreover, legumes are rich in carbohydrates, constituting up to 60% of their dry weight, providing a source of complex and energizing carbohydrates (Table 1). The starch found in legumes is metabolized at a slower rate compared to starch derived from tubers and cereal. This makes legumes suitable for individuals at high risk of acquiring diabetes and diabetic patients as they have a low glycemic index that aids in blood glucose control (Khalid and Elharadallou, 2013). Legumes also contain a significant amount of dietary fiber, accounting for up to 37% (Table 1) and providing both soluble and insoluble fiber (Kouris-Blazos and Belski, 2016). Diets rich in dietary fiber are associated with various health benefits, including prevention, and possible treatment of conditions such as hemorrhoids, certain cancers, diabetes, obesity, and constipation (Tamang et al., 2016).

Legumes are an excellent source of high-quality protein, comprising approximately 20～40% of their composition and often rich in lysine, an essential amino acid (Table 1). The notable protein content in legumes is attributed to the nitrogen-fixing bacteria present in their roots, which convert nitrogen gas into ammonium, a usable form for protein synthesis in plants (Maphosa and Jideani, 2017). With the exception of soybeans (28%) (Table 1), legumes generally have low to no cholesterol and only 5% of their calories come from fat (Messina, 1999). The fats in legumes primarily consist of mono- and polyunsaturated fatty acids (PUFA), while saturated fatty acids are absent (Maphosa and Jideani, 2017). Since the human body cannot synthesize these essential PUFAs crucial for health, they must be obtained through diet (FAO, 2016). Table 1 below provides the proximate composition (%) of various underutilized legumes.

SOURCE OF PROTEIN HYDROLYSATE

Plant proteins are essential components utilized in various meal preparations. They not only serve as sources of energy and amino acids but also provide nutritional value, influencing the physical and chemical characteristics of food (Etemadian et al., 2021). Pulses have been included in traditional meals worldwide for many years. Despite there being approximately 1,000 different legume species, only around 20 are extensively cultivated (Singh et al., 2022). The genetic resources of these underutilized crops are rapidly diminishing in their native environments, resulting in their increasing rarity worldwide. A paradigm shift is necessary to sustainably cultivate, exploit, and consume these species, moving away from their current state of neglect (Foyer et al., 2019). Research has shown that protein from hydrolyzed plants exhibits slightly higher functional and physiological qualities compared to crude protein (Ashaolu et al., 2017; Coscueta et al., 2019). The identification of new and affordable protein sources from vast plant resources has become crucial to meet the growing demand for protein (Prakash et al., 2001). As a result, several species, including Bambara groundnut (*Vigna subterranea* L.), Lablab (*Lablab purpureus* L.), winged bean (*Psophocarpus tetragonolobus* L.), kersting's groundnut (*Kerstingiella geocarpa* Harms), sword and jack bean (*Canavalia* spp.), rice bean (*Vigna angularis* L.), pigeon pea (*Cajanus cajan* L.), marama bean (*Tylosema esculentum* L.), lima bean (*Phaseolus lunatus* L.), mung bean (*Vigna mungo* L.) and African yam bean (*Sphenostylis stenocarpa* Harms) (Popoola et al., 2019), have been discovered.

PROTEIN HYDROLYSATE FROM UNDERUTILIZED LEGUMES

Protein hydrolysates are commonly employed as additives in food and feed production, as they possess the ability to modify various protein properties (Ward, 2011). Their beneficial impact on both food products and human health has made protein hydrolysates of great interest to the food industry (Sandberg, 2011). To produce protein hydrolysates, proteins are initially broken down into peptides of varying sizes. The resulting bioactive peptides

Table 1. Proximate composition of the legumes

Legumes	Composition (%)						References
	Moisture	Ash	Lipid	Fiber	Protein	NFE	
Soybean (*Glycine max*)	8.07	4.29	28.2	5.44	37.69	16.31	Etiosa et al., 2017
Jack bean (*Canavalia ensiformis*)	7.24	3.88	5.25	7.14	25.31	51.36	Solomon et al., 2018
Winged bean (*Psophocarpus tetragonolubus* L.)	10.21	3.52	18.12	11.47	27.81	28.87	Wan Mohtar et al., 2014
Pigeon pea (*Cajanus cajan* L.)	8.00	3.20	1.70	2.50	21.00	63.60	Mohammed Eltayeb et al., 2010
Lablab (*Lablab purpureus* L.)	8.47	3.50	1.02	1.21	23.95	61.86	Hossain et al., 2016
Lima bean (*Phaseolus lunatus* L.)	9.58	3.89	1.76	5.64	30.18	58.53	Chel-Guerrero et al., 2012
Mung bean (*Vigna mungo* L.)	10.21	3.02	1.53	4.95	23.84	56.43	Brishti et al., 2017
Bambara groundnut (*Vigna subterranea* L.)	7.22	3.76	6.28	5.48	20.44	52.80	Anhwange and Atoo, 2015

NFE, nitrogen-free extract.

typically consist of 2∼20 amino acid units, although this range may vary under certain circumstances (Shahidi and Ambigaipalan, 2019). Leguminous plants play a crucial role in both human and animal diets. Legumes, as the richest sources of plant protein, fulfill approximately 10% of the world's total protein dietary requirements (Soetan and Adeola, 2018). However, only a few legumes have been fully used to their potential (Wikandari et al., 2020). Hydrolysates and peptides derived from legumes exhibit a wide range of biological effects, including anticancer, antihypertensive, anti-inflammatory, hypolipidemic, antioxidant, and immunomodulatory properties (Matemu et al., 2021).

BIOACTIVITY OF PEPTIDES FROM UNDERUTILIZED LEGUMES' PROTEIN HYDROLYSATE

Antioxidant activity

The food sector has shown a growing interest in natural antioxidants derived from plant sources, driven by consumer preferences and concerns regarding synthetic antioxidants (Park et al., 2008). Protein hydrolysates, particularly those containing peptide-based antioxidants from natural sources, have gained attention as potential natural antioxidants. These hydrolysates possess characteristics such as low molecular weight, high activity, easy absorption, and cost-effectiveness (Sarmadi and Ismail, 2010). The antioxidant defense mechanism of the resulting peptides is determined by their ability to donate hydrogen, electrons, chelate metal ions, and scavenge free radicals (de Oliveira et al., 2015). Understanding the most potent peptides in terms of their sequence and size can provide insights into their fundamental mode of action, validating claims regarding the structure-function relationship (Matemu et al., 2021). In a study by Zainol et al. (2020), Jack bean protein hydrolysate prepared a hydrolysis time of 120-min exhibited the highest level of 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, hydroxyl radical inhibition, superoxide radical scavenging, and ferric reducing action, making it a promising antioxidant agent.

Antimicrobial activity

Antimicrobial activity refers to the ability of certain agents to inhibit the reproduction of bacteria, restrict the formation of microbial colonies, and sometimes cause the death of microorganisms (Elmogahzy, 2019). When the activity is sufficient to cause germicidal effects, it is described as cidal or fatal, and if it acts to suppress the growth of bacteria, it is referred to as bacteriostatic (Elmogahzy, 2019). Protein hydrolysates have been found to exhibit antimicrobial activity. They can stimulate the microbial autolytic system, a common characteristic among them (Taha et al., 2013). In a study by Zainol et al. (2020), Jack bean protein hydrolysate effectively inhibits the growth of *Pseudomonas aeruginosa*. This study unveils promising new properties of well-known antibacterial peptides, suggesting their potential use as food preservatives (Heymich et al., 2021).

Antihypertensive activity

The antihypertensive properties of certain biopeptides are attributed to the angiotensin I-converting enzyme (ACE), which hydrolyzes angiotensin I to produce the potent vasoconstrictor angiotensin II (Wan Mohtar et al., 2014). Several researches have been done on the enzymatic hydrolysis of protein-rich materials obtained from legumes, including winged bean (Wan Mohtar et al., 2014), pigeon pea (Olagunju et al., 2021), mung bean (Sonklin et al., 2020), Bambara groundnut (Arise et al., 2016), lima bean (Chel-Guerrero et al., 2012), and soybean (Daliri et al., 2019), to produce ACE inhibitory peptides. Natural ACE inhibitors have gained interest due to potential complications associated with synthetic ACE inhibitors, such as skin rashes, coughing, and taste disturbances. Consequently, several studies are focused on the isolation and production of ACE-inhibitory peptides from various food proteins (Boschin et al., 2014).

Foaming ability

The ability of hydrolyzed peptides to foam is influenced by their molecular size and degree of hydrolysis. Peptides with higher molecular weight generally contributed to the stability of foam (Peighambardoust et al., 2021). When proteins are hydrolyzed and denatured, their structural alterations, and unfolding often enhance their foaming properties, allowing them to efficiently reorganize and relocate at the air-water interface (Klompong et al., 2007). Hydrolysates with more hydrophobic regions and unfolded structures are effectively absorbed at the air-water interface, leading to increased foaming capabilities (Peighambardoust et al., 2011). The pH level also plays a role in the surface hydrophobicity of peptides, affecting the adsorption of proteins at the air-water interface and subsequently influencing their foaming ability (Peighambardoust et al., 2021). Polanco-Lugo et al. (2014) reported that lima bean protein hydrolysate exhibited foaming ability ranging from 200% to 145% at pH values of 2, 4, 6, 8, and 10 during their experiments.

Emulsifying property

Protein hydrolysates and bioactive peptides can possess surface-active properties that contribute to stabilizing oil-in-water emulsions, facilitated by their hydrophobic and hydrophilic groups (Peighambardoust et al., 2016). The emulsifying abilities of these peptides rely on their inter-

action with oil droplets at the surface during homogenization, forming a protective layer that hinders droplet coalescence (Fasihnia et al., 2018). Klompong et al. (2007) demonstrated that the degree of hydrolysis has a significant impact on the emulsifying activity of peptides and the resulting stability of the emulsion. This finding aligns with the research conducted by Zheng et al. (2020), which demonstrates that increasing the hydrolysis time of mung bean protein hydrolysate had a detrimental effect on its emulsifying properties.

EXTRACTION OF PROTEIN HYDROLYSATE

Protein extraction can be performed using a dry or wet method. The dry fractionation technique involves separating fractions based on particle shape, density, and size using air classification and grinding techniques (Ferreira et al., 2022). Dry fractionation is considered a significant approach for extracting protein from legume seeds and serves as a viable alternative to water extraction, as it preserves protein functionalities (Jebitta et al., 2021). In contrast, wet processing yields flour with higher protein purity. Common methods used for legume seeds and fractions include enzymatic extractions, acid/alkaline treatments, solvent extraction, and the utilization of ultra-filtration membranes (Jebitta et al., 2021). This method offers the advantage of producing isolated proteins that are highly soluble and stable (Franca-Oliveira et al., 2021). Successful extraction yield of aqueous protein and preservation of functionality depends on important parameters such as the protein's bioaffinity, the electrical potential across the phase system, molecular size, and hydrophobicity of the extraction phase (Asenjo and Andrews, 2011).

Chemical-assisted extraction method

Chemical-assisted extraction methods are categorized according to the solvents utilized, which include organic solvents, acids, alkali, and water (Franca-Oliveira et al., 2021). While the effectiveness of the methodology largely depends on the composition of the protein sample, processing conditions have also been found to impact protein recovery (Lee et al., 2017). Aqueous extraction is a popular approach as isolated proteins exhibit high solubility and stability in water. Furthermore, the method offers advantages such as a simple procedure and low cost (Chen et al., 2019).

The selection of alkalis such as KOH and NaOH, alkali concentration, temperature, molecular size of the plant substrate, plant substrate-to-solvent ratio, and extraction time are all crucial factors in obtaining maximum protein extraction yield (Zhang et al., 2014). Chemical extraction techniques are widely used for plant protein extraction due to the improved bioavailability and digestibility of the extracted proteins (Ampofo and Ngadi, 2022). Organic solvents, such as ethanol, acetone, and butanol, are necessary for extracting proteins that contain hydrophobic or aromatic amino acid residues with or without nonpolar characteristics (Cui et al., 2017). However, the usage of organic solvents in protein extraction results in a lower yield of extracted protein (approximately 50%), lower protein quality due to amino acid loss and denaturation, as well as the generation of lysinoalanine. Moreover, this process is time-consuming, energy-intensive, and environmentally unfriendly (Deleu et al., 2019).

Enzyme-assisted extraction technique

Enzyme-assisted extraction (EAE) is a sustainable technique that utilizes enzymes to degrade key components of the cell wall, such as pectin, cellulose, and hemicellulose, resulting in the disruption of the wall and the release of cellular proteins (Pojić et al., 2018). Proteases then break down high molecular weight cell proteins into smaller, highly soluble fragments, facilitating more efficient extraction (Kumar et al., 2021). EAE is considered a green extraction method with significant potential due to its low environmental impact and moderate extraction conditions (Cheng et al., 2015).

Furthermore, this approach uses enzymes with great specificity and efficiency (Harun and Hamid, 2021), resulting in products that are more suitable for human consumption and purer (Franca-Oliveira et al., 2021). These methods improved the essential amino acid score, protein ratio, amino acid profile, and the potential for bioactive peptide production, making them suitable for protein extraction with remarkable functional and nutritional properties (Franca-Oliveira et al., 2021). Several parameters, such as time, particle size, types of enzymes, incubation temperature, enzyme concentration, pH, and liquid to solid ratio, affect the yield and efficiency of the EAE process (Liu et al., 2016).

Previous studies have reported that protein hydrolysates from soybean and lima bean, extracted using bromelain, and alcalase-flavourzyme enzyme systems, show a higher degree of hydrolysis (Table 2). Moreover, protein hydrolysates extracted from other legumes such as Jack bean, Lablab, Bambara groundnut, and mung bean have demonstrated antioxidant properties, particularly against DPPH radicals. Furthermore, protein hydrolysates from the winged bean and pigeon peas have been shown to express a potent ACE inhibitory activity (Table 2). Table 2 below indicates the enzymatic hydrolysis of several legumes' proteins.

Table 2. Enzymatic hydrolysis of the protein derived from legumes

Sample	Enzyme	Outcome	References
Soybean	Bromelain	Highest degree of hydrolysis	Utami et al., 2019
Jack bean	Alcalase	It exhibits the highest inhibition against hydroxyl radicals and DPPH, making it an excellent antioxidant agent for superoxide radical scavenging and ferric-reducing activity. Additionally, it demonstrates successful inhibition against *Pseudomonas aeruginosa*	Zainol et al., 2020
Winged bean	Alcalase, bromelain, flavourzyme, papain	Papain was reported to be the most effective protease for producing winged bean seed hydrolysate with significant ACE inhibitory activity	Wan Mohtar et al., 2014
Pigeon pea	Thermoase	Able to inhibit activities of renin and ACE inhibitory activity	Olagunju et al., 2021
Lablab	Pepsin, alcalase, trypsin	Protein hydrolysate and isolates from *Lablab purpureus* demonstrated good antioxidant activity	Sipahli et al., 2022
Lima bean	Alcalase-flavourzyme, pepsin-pancreatin	The alcalase-flavourzyme hydrolysates showed the highest degree of hydrolysis. Meanwhile, the pepsin-pancreatin hydrolysates exhibited the highest inhibitory activities against DPP-IV	Castañeda-Pérez et al., 2021
Mung bean	Ficin	Effectively inhibit the oxidation of the lipids in sunflower oil and the sunflower oil-in-water emulsion	Zheng et al., 2020
Bambara groundnut	Alcalase, trypsin, pepsin	Possess antioxidant properties against a DPPH radical	Arise et al., 2016

DPPH, 2,2-diphenyl-2-picrylhydrazyl; ACE, angiotensin I-converting enzyme; DPP-IV, dipeptidyl peptidase IV.

USAGE OF PROTEOLYTIC ENZYMES

Enzymes play a crucial role in both anabolic and catabolic pathways, serving as biocatalysts that lower the activation energy of biological reactions (Sandoval and Hyster, 2020). Proteolytic enzymes specifically catalyze the breakdown of peptide linkages between amino acid residues in proteins. These enzymes are commonly referred to as proteases or peptidases and fall under the category of hydrolase enzymes (Dhillon et al., 2017). Proteolytic enzymes can also be classified based on other factors (Dhillon et al., 2017).

Moreover, proteases can be further categorized as endopeptidases or exopeptidases based on the site of enzyme action. Exopeptidases catalyze the hydrolysis of peptide bonds near the N- or C-terminal ends of the substrate, while endopeptidases break down peptide bonds within the polypeptide chain, both near and far from the ends (Mótyán et al., 2013).

Proteases derived from plant-based sources, such as papain from pineapple and bromelain from papaya, have found applications in the production of protein hydrolysates, which possess high nutritional value and are used in baking and as meat tenderizers (Dhillon et al., 2017; Sharma et al., 2019).

PURIFICATION OF PROTEIN HYDROLYSATE

There are numerous protein purification techniques available that can be combined to create an appropriate purification strategy (Labrou, 2014). Typically, protein purification involves multiple steps as proteins are rarely purified in a single step, even if a specific biological property is targeted (Wilken and Nikolov, 2012). In the initial stages of the purification scheme, low-resolution, and high-capacity techniques are employed when larger amounts of protein are present, while higher-resolution, and lower-capacity methods are used when only small amounts of protein are available. Techniques such as two-phase partition systems and fractional precipitation are commonly used for low-resolution protein purification (Rosa et al., 2011). In broader-scale protein purification, ion exchange chromatography, western blotting, magnetic bed separation, size exclusion chromatography, and other methods are employed (Kumar and Nayak, 2019).

Ion exchange chromatography

Ion exchange chromatography is a separation technique that involves the use of a charged particle-packed column. An anion exchanger refers to a positively charged column that binds negatively charged peptides, while a negatively charged column is known as a cation exchanger as it binds positively charged peptides (Aluko, 2018). Similar to other column-based liquid chromatography methods, this technique uses both stationary and mobile phases. The stationary phase is an aqueous buffer system where the mixture to be resolved is introduced, while the mobile phase is an inert organic matrix chemically derivatized with ionizable functional groups that carry a displaceable oppositely charged counterion (Cummins et al., 2017). Peptides that are weakly bound to the column will elute first, followed by those that are strongly bound (Aluko, 2018).

In natural products, the charged organic molecules are often represented by protonated bases, such as alkaloids, or deprotonated acids, such as fatty acids, or derivatives of amino acids. Most naturally occurring substances that are separated using ion exchange chromatography possesses a functional group capable of ionization, such as a phenolic proton, carboxylic acid, or alkaloid (Dragull and Beck, 2012).

Size exclusion chromatography

Size exclusion chromatography, also known as gel filtration chromatography, is a method used for separating molecules based on their sizes (Duong-Ly and Gabelli, 2014). The technique involves a column filled with a matrix, usually in the form of beads, which contains both an internal volume (liquid inside the beads) and an external volume (liquid between the beads) (Walls and Loughran, 2017). When a sample is applied to the column, molecules larger than the pores of the matrix are excluded from entering the beads' internal volume, causing them to elute quickly. On the other hand, molecules that are similar, or intermediate in size equilibrate with both the external and internal liquid volumes, resulting in slower elution through the column (Walls and Loughran, 2017).

Gel filtration chromatography is particularly suitable for biomolecules that are sensitive to pH changes, metal ion concentrations, cofactor concentrations, or harsh environmental conditions. It can be used after ion exchange chromatography, as the final separation is typically independent of the buffer composition (Wang et al., 2017).

Reversed-phase liquid chromatography

Reversed-phase liquid chromatography (RPLC) is a highly efficient technique within high-performance liquid chromatography that utilizes a nonpolar stationary phase and a polar mobile phase comprises of at least one water-miscible organic solvent and water (Soliven et al., 2013). By diluting the mobile phase with water, its solvent strength is increased, altering selectively by reducing the cohesive energy of the binary mobile phase and promoting stronger solute-solvent interactions compared to pure water (Poole and Lenca, 2017). Hydrophobicity is the primary parameter used to distinguish peptides with less than 30～40 amino acid residues, and the retention times of peptides with known compositions can be accurately predicted (Conlon, 2007).

RPLC offers numerous advantages, including its compatibility with aqueous samples, gradient elution capability, the ability to separate ionic, nonpolar, and polar compounds within a single run, a wide range of commercially available stationary phases, and its extensive application in various fields (García-Alvarez-Coque et al., 2016).

Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) has emerged as a crucial bioanalytical technology for the comprehensive characterization of individual proteins (O'Connor and Cummins, 2017). It is a high-resolution chromatography method that operates by weakly interacting hydrophobic ligands on the stationary phase with hydrophobic regions on the surface of proteins' tertiary structure (Rackiewicz et al., 2017). In HIC, solutes (proteins) are adsorbed and isolated on a stationary solid phase (a two-dimensional system) consisting of immobilized hydrophobic groups (Jennissen, 2001).

HIC is frequently used as a purification technique for proteins that lack affinity tags, such as his-tags, and when ion exchange chromatography is not effective in separating or purifying proteins (O'Connor and Cummins, 2017).

CHARACTERIZATION OF AMINO ACID FROM UNDERUTILIZED LEGUMES' PROTEIN HYDROLYSATE AND MOLECULAR WEIGHT OF AMINO ACID DERIVED FROM UNDERUTILIZED LEGUMES' PROTEIN HYDROLYSATE

Peptide chain length plays a significant role as it partially influences various functional characteristics (Wasswa et al., 2007). When proteins are hydrolyzed into shorter peptides, the molecular weight distribution changes, and some hydrophobic groups that were originally folded within the intact protein structure become exposed to the aqueous phase (Muhamyankaka et al., 2013). The hydrolysis of proteins affects their molecular weight, which is also associated with the bioactivity of protein hydrolysates (Yang et al., 2011). The molecular weight of protein hydrolysates from underutilized legumes can vary, ranging from 0.59 to 200 kDa depending on the hydrolysis conditions (Table 3). Table 3 shows the molecular weight information of amino acids derived from protein hydrolysates of underutilized legumes.

AMINO ACID COMPOSITION IN PROTEIN HYDROLYSATE OF UNDERUTILIZED LEGUMES

All 20 amino acids can be synthesized by certain organisms, such as fungi, plants, and bacteria. However, for monogastric mammals including humans, only 11 out of the 20 amino acids can be synthesized, while the remaining nine amino acids, known as essential amino acids, must be obtained through the diet (Anjum et al., 2017). Moreover, as amino acids are the fundamental components of proteins, any molecule with both amino and carboxylic acid functional groups is considered an amino

Table 3. Molecular weight of amino acid derived from protein hydrolysate of the underutilized legumes

Source of protein hydrolysate	Hydrolysis condition	Molecular weight of amino acid (kDa)	References
Soybean	Before being added to the protein suspensions, the pepsin enzyme solution at 2 U/mg of protein activity levels was warmed to 37°C The enzyme and protein combinations were shaken at 140 rpm in a water bath for 30, 60, 90, 120, 150, 180, and 1,440 min	200 to 6.5	Edwards et al., 2020
Pigeon pea	Enzymatically hydrolyzed with pepsin+pancreatin (pH 2.0 for pepsin followed by 7.5 for pancreatin at 37°C); alcalase (pH 8.0, 50°C) and pancreatin (pH 7.5, 37°C)	Pigeon pea protein hydrolysate hydrolyzed with alcalase had a peptide size range from 3.17 to 0.59 kDa Pigeon pea protein hydrolysate hydrolyzed with pancreatin had a peptide size range from 4.89 to 0.59 kDa	Olagunju et al., 2018
Lablab	Three proteases used were pepsin (pH 2, 37°C), alcalase (pH 8, 50°C), and trypsin (pH 8, 37°C)	198 to 14 kDa	Sipahli et al., 2022
Lima bean	Enzymatically hydrolyzed with pancreatin (pH 7.5) and pepsin (pH 2) at 37°C	34.6 to 7.1 kDa	Polanco-Lugo et al., 2014
Mung bean	The first aliquot was incubated with ficin (2%, w/w) at pH 5.7 and 65°C, and the second one with bromelain (2%, w/w) at pH 7.0 and 55°C Hydrolysis was done for 60, 120, 180, 240, and 300 min	All ficin hydrolysates with molecular weights between 50 and 15 kDa showed a clear transition from high molecular weight bands to low molecular weight units All bromelain-treated hydrolysates had the distinct and uniform band about 15 kDa, despite the hydrolysis time	Zheng et al., 2020
Bambara groundnut	Enzymatically hydrolyzed with pancreatin (pH 8.5, 37°C)	80 to 30	Mune, 2015
Jack bean	NA		
Winged bean	NA		

NA, not available.

acid. Amino acids play a vital role as biological building blocks as they can combine through amide linkages, connecting the NH2 of one amino acid with the COOH of another, thus forming long chains (Aremu et al., 2017).

The functional characteristics of peptides can vary depending on their amino acid composition (He et al., 2013). It has been observed that acidic amino acids such as aspartic acid and glutamic acid, which are acidic amino acids, possess strong antioxidant properties due to their ability to scavenge free radicals and maintain an excess of electrons (Udenigwe and Aluko, 2012). Consequently, the higher levels of Glu and Asp found in the protein hydrolysates from soybean, pigeon pea, lima bean, mung bean, and Bambara groundnut suggest that these legumes may have stronger antioxidant properties (Table 4). Fathollahy et al. (2021) demonstrated that a higher content of hydrophobic amino acids (HAAs) such as Cys, Leu, Ala, Pro, Met, Val, Phe, and Ile in protein derived from Persian lime seeds enhances protein stability and aids in lipid dispersion in emulsions. Additionally, a significant concentration of HAAs can enhance antioxidant activity by increasing the solubility of peptides in fat (Xie

Table 4. Amino acid composition from protein hydrolysates of legumes

Legumes	Composition of amino acid (g/100 g)																	
	Asp	Glu	Arg	Lys	His	Ala	Ile	Leu	Met	Phe	Pro	Val	Trp	Cys	Ser	Thr	Tyr	Gly
Soybean[1)]	12.10	19.70	7.40	6.60	2.50	4.40	3.70	7.50	1.20	4.90	6.40	3.90	1.30	2.20	5.20	3.90	3.10	4.30
Pigeon pea[2)]	10.77	20.45	7.24	7.74	4.78	4.12	3.82	8.88	0.84	8.87	4.92	3.94	0.23	0.65	6.33	3.95	2.79	3.38
Lablab[3)]	0.856	2.98	0.005	2.64	0.95	0.57	0.75	1.80	0.21	2.18	0.43	0.74	0.15	0.13	0.18	0.17	0.15	0.43
Lima bean[4)]	12.30	12.50	7.30	5.90	1.40	9.00	3.90	8.40	0.60	5.00	3.70	6.30	0.80	0.10	6.30	6.40	2.80	7.30
Mung bean[5)]	8.76	14.24	5.21	5.18	2.07	2.70	3.17	5.64	1.19	4.62	2.94	4.25	0.00	0.51	4.01	2.32	2.11	2.10
Bambara groundnut[6)]	8.31	20.09	7.15	9.33	4.93	5.04	4.63	9.28	0.09	5.68	4.95	5.40	0.23	0.00	4.36	2.91	4.49	3.13
Jack bean	NA																	
Winged bean	NA																	

[1)]Claessens et al., 2007. [2)]Olagunju et al., 2021. [3)]Bai-Ngew et al., 2021.
[4)]Polanco-Lugo et al., 2014. [5)]Liu et al., 2022. [6)]Thammarat et al., 2015.
NA, not available.

et al., 2019). Moreover, certain HAAs such as Pro, Ile, Trp, Tyr, Val, and Phe are often associated with ACE inhibitory effects (Polanco-Lugo et al., 2014). Intorasoot (2013) discovered that amino acids with positive charges, such as histidine and lysine, are among those that can inhibit microorganisms. Methionine and cysteine, on the other hand, are present in small amounts in pulse proteins (Palupi et al., 2022). Table 4 presents amino acid composition of protein hydrolysate from various legumes.

PREDICTING AMINO ACID MODEL FROM AN AMINO ACID SEQUENCE OF LEGUMES' PROTEIN HYDROLYSATE

The remarkable diversity of molecular activities exhibited by naturally occurring proteins is made possible by their precisely folded three-dimensional (3D) structures, which are determined by the specific sequences of amino acids encoded in their genes (Kuhlman and Bradley, 2019). Understanding the relationship between protein sequence, structure, and function has become crucial in modern biomedical research (Pearce and Zhang, 2021). Nuclear magnetic resonance spectroscopy and X-ray crystallography are the two primary experimental methods used to determine protein structures (Deng et al., 2018). However, there have been no studies attempting to predict the 3D structure of proteins derived from underutilized legumes.

In a recent study by Wang et al. (2021), two novel ACE inhibitory peptides (VERGRRITSV and FVIEPDITPA) were successfully isolated and identified from the hydrolysate of walnut glutelin-1. The study demonstrated that these ACE inhibitory peptides derived from walnut meal exhibited significant activity in inhibiting ACE both *in vivo* and *in vitro*. This finding suggests that these peptides may have potential applications in the development of antihypertensive foods and medications.

POTENTIAL APPLICATION OF PROTEIN HYDROLYSATE AS FUNCTIONAL FOODS

The increasing demand for functional foods and nutraceuticals reflects a growing awareness of the impact of diet on health (Soumya et al., 2021). Functional foods are defined as natural or processed foods that offer additional health benefits and disease prevention capabilities beyond basic nutrition (Soumya et al., 2021). Protein hydrolysates derived from high protein sources have gained attention due to their wide range of therapeutic effects and significant bioactivity (Nasri, 2017). They are also considered safer and milder for consumers. Additionally, studies have shown that peptides and protein hydrolysates with lower molecular weights are less allergenic compared to the parent proteins they are derived from (Peighambardoust et al., 2021). Enzymatic hydrolysis has been found to enhance the functionality of protein hydrolysates (Liceaga and Hall, 2019), making them more effective in various applications. There is a growing interest in using protein hydrolysates as ingredients in unique formulations (Nasri, 2017).

The applications of proteins in the food sector can be optimized through minimal hydrolysis. This review focuses on exploring the functional characteristics of protein hydrolysates and their potential applications. Research has demonstrated the efficacy of dark red kidney bean (*Phaseolus vulgaris* L.) hydrolysates, which are abundant in peptides and polyphenols, in inhibiting oxidation in plain yogurt products during storage at room temperature for three days. These hydrolysates exhibit higher antioxidative stability compared to ascorbic acid (Sarker et al., 2020). Another study by Gomes and Kurozawa (2021) have shown the use of rice protein hydrolysate as an encapsulating matrix in linseed oil microparticles. This application enhances the stability of lipids containing unsaturated fatty acids. Furthermore, land-animal protein serves as a valuable source for obtaining antioxidant hydrolysates or peptides due to their higher bioavailability and content of essential amino acids (Wu and Chen, 2022).

Al-Shamsi et al. (2018) conducted a study demonstrating that camel milk protein hydrolysate exhibits favorable functional properties and remarkable antioxidant capacity, both *in vitro*, and in real food systems. These findings suggest the potential application of camel milk protein hydrolysate functional foods. Another study conducted by Sun et al. (2010) also concludes that deboned chicken-residue hydrolysates effectively reduce oxidation and Maillard reaction products in Cantonese sausage without causing significant changes to the sensory qualities of the sausage.

However, there is currently limited information available regarding the potential application of protein hydrolysate derived from underutilized legumes as functional foods, despite their nutritional value, high satiety value, pleasant taste, and affordability (Fasoyiro et al., 2010). In a study conducted by Akoja et al. (2017), it was found that there is a considerable rise in the protein content of maize-based snacks (Kokoro) significantly increased with the addition of pigeon pea protein hydrolysate (Table 5). This increase in protein content may be attributed to the high protein content of pigeon peas, as previously reported in other studies (Adegunwa et al., 2015).

Moreover, the addition of Lablab protein hydrolysate to apple juice has been shown to significantly reduce the levels of oxidative compounds in the juice (Table 5) (Roy et al., 2022). This antioxidant effect may be attributed to the presence of bioactive peptides with antioxidant ac-

Table 5. Application of protein hydrolysates of the legumes in food products

Source of protein hydrolysate	Types of food tested	Influence on food product	References
Soybean	Sausages	Enhanced gel forming and emulsion stability properties	Kamani et al., 2019
	Beef burger	Improved water holding capacity of patties	Carvalho et al., 2017
Pigeon pea	Maize-based snack (Kokoro)	The results of the proximate analysis showed a significant increase in the protein content of the snacks as the protein hydrolysate content in the final product increased	Akoja et al., 2017
Lablab	Semi-dried rice noodle	Lablab protein hydrolysate (at a concentration of 200 mg/mL) not only improved the color of the noodles but also reduced the cooking time. Furthermore, when compared to the control noodle without any additive, the addition of Lablab protein hydrolysate significantly increased the shelf life of the noodles, extending it by more than three times	Bai-Ngew et al., 2021
	Apple juice	Significantly reduced the production of oxidative compounds in apple juice relative to protein concentrates and control	Roy et al., 2022
Lima bean	Pasta	After the processing of pasta, the addition of *Phaseolus lunatus* hydrolysates at 5% and 10% concentrations resulted in the retention of the majority of their antioxidant activity. Although a slight reduction in antioxidant activity was observed, it was determined that neither leaching nor inactivation during pasta cooking led to the loss of bioactivity in the hydrolysates	Drago et al., 2016
Mung bean	NA		
Bambara groundnut	NA		
Jack bean	NA		
Winged bean	NA		

NA, not available.

tivity that are generated during the enzymatic hydrolysis of Lablab protein (Roy et al., 2020). It was also demonstrated that pasta fortified with lima bean protein hydrolysate retains a significant portion of its activity even after processing (Table 5) (Drago et al., 2016). This could be attributed to the lower degree of hydrolysis of lima bean protein hydrolysate, which allows the majority of the protein's secondary structure to remain intact during the hydrolysis process. This, in turn, enhances the protein-water interaction in the final product (Drago et al., 2016). Table 5 provides an overview of the application of legume protein hydrolysates as functional food ingredients in various food products.

CONCLUSION

The popularity of plant-based whole foods and plant-based protein products is on the rise, and this trend is expected to continue due to their reported advantages over animal protein food products in terms of human health and environmental sustainability. Plant proteins offer a wide range of essential nutrients such as dietary fiber, vitamins, minerals, and phytochemicals, which may not be as abundant in meat proteins. Consuming a variety of plant proteins can ensure a diversified nutrient intake. Furthermore, plant proteins are generally low in cholesterol and saturated fat, unlike certain meats, particularly red, and processed meats, which can be high in both. Opting for plant proteins can promote cardiovascular health, as a high intake of saturated fat and cholesterol has been linked to an increased risk of heart disease.

In addition to the health benefits, choosing plant-based protein sources can have a lower environmental impact compared to raising animals for meat production. Animal agriculture contributes significantly to greenhouse gas emissions, deforestation, water pollution, and habitat destruction. By selecting plant-based protein sources, consumers can reduce the ecological footprint associated with food production.

This review focuses on protein hydrolysates derived from underutilized legumes, which have the potential to serve as future functional foods. Protein hydrolysates from these legumes have demonstrated important functions such as antioxidant, antimicrobial, antihypertensive, foaming, and emulsifying properties. These findings shed light on the potential use of these peptides in the development of functional food products. However, it is important to note that different legumes vary in terms of protein quality, and in many cases, legume proteins may be deficient in certain essential amino acids. As a result, food experts are actively studying various methods to enhance the quality of legume proteins and address these limitations.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: ATA, MKZ. Analysis and interpretation: ATA. Data collection: ATA. Writing the article: ATA. Critical revision of the article: MKZ. Final approval of the article: all authors. Statistical analysis: ATA. Obtained funding: MKZ. Overall responsibility: all authors.

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