ผลของการไฮโดรไลซิสบางส่วนด้วยโปรตีเอสต่อสมบัติทางโภชนาการ สมบัติทางโครงสร้าง สมบัติเชิงหน้าที่ และฤทธิ์ทางชีวภาพของกากถั่วลูกไก่เหลือทิ้งจากกระบวนการผลิตน้ำนมถั่วลูกไก่

Effect of Partial Hydrolysis by Proteases on Nutritional, Structural, Functional, and Bioactive Properties of Chickpea Meal Wasted from Chickpea Milk Production

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บทคัดย่อ

กากถั่วลูกไก่ (CPM) ซึ่งเป็นผลพลอยได้จากการผลิตน้ำนมถั่วลูกไก่ มีปริมาณโปรตีน ร้อยละ 18 โดยน้ำหนักแห้ง การไฮโดรไลซิสบางส่วนมีประสิทธิภาพในการปรับปรุงสมบัติของโปรตีนพืช การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของการไฮโดรไลซิสบางส่วนต่อสมบัติของ CPM โดยใช้อัลคาไลน์โปรตีเอส 50 U/g substrate ที่พีเอช 9.0 หรือนิวทรัลโปรตีเอส 48 U/g ที่พีเอช 7.0 อุณหภูมิ 45 องศาเซลเซียส เป็นเวลา 90 นาที เพื่อผลิตไฮโดรไลเซท CPH-AP และ CPH-NP ซึ่งมีค่าระดับการย่อยสลาย ร้อยละ 24 และร้อยละ 14 ตามลำดับ ผลการศึกษาพบว่าการไฮโดรไลซิสบางส่วนทำให้น้ำหนักโมเลกุลของโปรตีนลดลง โครงสร้างทุติยภูมิของโปรตีนเปลี่ยนแปลง แต่ไม่ส่งผลต่อองค์ประกอบกรดอะมิโนและค่าการย่อยได้ของโปรตีน ในขณะที่ความสามารถในการละลายของโปรตีนเพิ่มขึ้น CPH-AP มีฤทธิ์ต้านอนุมูลอิสระที่วัดด้วยวิธี ORAC (924.34 µmol TE/g protein) ประมาณ 2 เท่าของ CPH-NP และ CPM CPH-AP และ CPH-NP มีฤทธิ์ยับยั้งเอนไซม์แองจิโอเทนซิน-คอนเวอร์ติง ร้อยละ 94.74 และร้อยละ 92.82 ตามลำดับ ด้านฤทธิ์ต้านการอักเสบ พบว่า CPH-AP สามารถยับยั้งการหลั่งไนตริกออกไซด์ และอินเตอร์ลิวิคิน-6 ได้ดีกว่า CPM 14 และ 7 เท่าตามลำดับ ผลการศึกษานี้แสดงให้เห็นว่าการไฮโดรไลซิสบางส่วนด้วยเอนไซม์สามารถเพิ่มศักยภาพของ CPM ในการเป็นส่วนผสมอาหารจากพืชที่ยั่งยืนและมีฤทธิ์ทางชีวภาพได้

คำสำคัญ: ถั่วลูกไก่ โปรตีนไฮโดรไลเสต การต้านอนุมูลอิสระ การต้านความดันสูง การต้านการอักเสบ

ABSTRACT

Chickpea meal (CPM), which is a by-product of chickpea milk production, contains 18% protein on dry basis. Partial hydrolysis is efficient in improving properties of plant proteins. This study aimed to determine the impact of partial hydrolysis ion properties of CPM. Hydrolysis was performed using alkaline protease at 50 U/g substrate at pH 9.0, or neutral protease at 48 U/g substrate at pH 7.0 at 45°C for 90 min to obtain the hydrolysates, CPH-AP and CPH-NP with degree of hydrolysis of 24 and 14%, respectively. It was found that partial hydrolysis reduced molecular weight and changed the secondary structure of protein but did not affect the amino acid composition and protein digestibility, while increased protein solubility. CPH-AP showed the highest antioxidant activity of 924.34 µmol TE/g protein, double that of CPH-NP and CPM, as assessed by oxygen radical antioxidant capacity. CPH-AP and CPH-NP exhibited strong angiotensin-converting enzyme inhibitory activities 94.74% and 92.82%, respectively. For anti-inflammatory properties, CPH-AP showed a 14-fold and 7-fold greater inhibition on nitric oxide and interleukin-6 than CPM. These findings revealed that enzymatic partial hydrolysis could improve the potential application of CPM as a sustainable, functional plant-based ingredient.

Keywords: chickpea, protein hydrolysate, antioxidation, antihypertension, anti-inflammation

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INTRODUCTION

The growing shift toward plant-based diets, driven by health and environmental concerns, has led to increased demand for plant-based products [1]. Chickpea (*Cicer arietinum* L.) has gained global popularity not only for its culinary use but also for its rich nutritional profile including high in protein, fiber, vitamins, minerals, and antioxidants. Owing to its superior nutritional quality, hypo-allergenicity to other plant protein sources [2-6], chickpea has become an alternative raw material for plant-based milk manufacture, from which chickpea meal (CPM) is the major by-product. It is roughly estimated that the amount of CPM could reach 30 – 40% of the amount of chickpea milk obtained in the production. Currently, chickpea meal is underutilized by chickpea milk manufacturers, as it is sold at a low-price for animal feeding and composting. Thus, CPM is a promising ingredient for plant-based food products or a raw material for protein extraction and further preparation of protein products.

Numerous studies have focused on the extraction, isolation, and characterization of chickpea protein, which serves as a valuable alternative plant protein source. Previous studies have explored various extraction techniques, such as alkaline solubilization, isoelectric precipitation, and enzymatic hydrolysis, to enhance protein yield and functionality [7]. In addition, a number of studies suggested the health benefits of enzymatically hydrolyzed dietary protein-based peptides [8, 9]. However, enzymatic hydrolysis may have potential drawbacks, including the development of off-flavors and bitterness due to the extensive breakdown of polypeptides. Over-hydrolysis may also reduce the functional properties of the protein, limiting its application in food formulations. It is proven that enzymatic partial hydrolysis of plant proteins can produce antioxidative hydrolysates, of which the degree of hydrolysis ranging from 10 to 30%, that have potential benefits on health [10].

Currently, the nutritional contents, biological activities, structural properties, and functional properties of protein hydrolysates derived from partial hydrolysis of chickpeas, particularly CPM, have not been extensively explored. Moreover, in most previous studies, chickpea protein hydrolysates and peptides were obtained by hydrolyzing the protein isolated from whole chickpea, rather than intact protein, which not only increases production cost at an industrial scale but also adds complexity to by-product management.

Therefore, this study aims to provide an in-depth exploration of protein hydrolysates and peptides obtained through partial enzymatic hydrolysis of CPM. Nutritional, structural, functional properties, as well as in vitro bioactivities of the obtained hydrolysates were examined, with an emphasis on their potential as functional ingredients with applications in the prevention and management of chronic diseases. The data obtained will be useful for the valorization of CPM as an ingredient with potential health benefits.

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MATERIALS AND METHODS

1. Materials

Chickpea meal was obtained from a local chickpea milk manufacturer in Bangkok, Thailand. The meal from 3 production batches was immediately collected after separating the chickpea milk, packed in sealed clear high-density polyethylene bags and kept frozen at -18°C prior to transporting them in an iced box to the laboratory where a sample of each batch was determined for nutritional composition. The chickpea meal from 3 batches were then pooled together and kept in a freezer until being thawed and used for experiments without any further preparation or modification. Protease enzymes, namely iKnowZyme® Alkaline Protease (AP) and iKnowZyme® Neutral Protease (NP) was obtained from Reach Biotechnology Co., Ltd. (Bangkok, Thailand). Both enzymes are microbial endopeptidases prepared from *Bacillus licheniformis* 2709 and *Bacillus subtilis* 1398, respectively, and are on the list of enzymes allowed for food processing by the Food and Drug Administration of Thailand [11]. The declared enzyme activity was ≥250,000 U/mL for AP and 0.8 U/g for NP. All chemical reagents used in this study are analytical grade and obtained from Sigma-Aldrich (St. Louis, Missouri, USA), unless stated otherwise.

2. Preparation of chickpea meal hydrolysates

Enzymatic hydrolysis of chickpea meal was conducted using commercial proteases AP or NP, following the modified method described by Shi and Tsou [12, 13]. Prior to hydrolysis, the thawed chickpea meal was dispersed in deionized water at a meal-to-water weight ratio of 2:1. The pH of the mixture was adjusted to 9.0 or 7.0, which is the optimum pH for AP and NP, respectively, by adding 1 N HCl or 1 N NaOH solution. Hydrolysis using AP was performed using an E:S ratio of 200 mg/kg dry material (50 U/g) while for NP, an E:S ratio of 600 mg/kg (48 U/g) was used. For both enzymes, the hydrolysis was performed in temperature-controlled water at 45°C for 90 min. These hydrolysis condition parameters for each enzyme were obtained from our preliminary optimization experiments to achieve the highest protein extraction yield (unpresented data). Upon completion, the hydrolysates were heated at 95°C for 10 min in a water bath to inactivate the enzyme, followed by cooling down immediately to room temperature in an ice bath and centrifuged at 10,000 ×g for 15 min at 4°C. The supernatants were neutralized and collected for freeze-drying to obtain dry hydrolysate pellets, which were sealed in aluminum foil sachets and stored in a desiccator until being used for analysis. The hydrolysis was performed in 3 replicates for each enzyme. The hydrolysates, including that prepared using AP (CPH-AP) and NP (CPH-NP), had degree of hydrolysis of 23.63 ± 0.44% and 13.53 ± 1.56%, respectively, as determined using the 2,4,6-trinitrobenzene sulfonic acid method [14].

3. Characterization of chickpea meal hydrolysates

Subsequent analyses were performed on the hydrolyzed samples, namely CPH-AP and CPH-NP, and compared with non-hydrolyzed chickpea meal (CPM) as a control.

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3.1 Nutritional properties

Proximate composition including protein, fat, ash and carbohydrates content was determined following the AOAC official methods [13]. Amino acid profile was analyzed using an amino acid analyzer (ARACUS, Membrapure, Hennigsdorf, Germany). Samples were hydrolyzed with 6 N HCl using a microwave extraction system (ETHOS X, Milestone Srl, Sorisole, Italy) under continuous stirring prior to analysis by cation exchange chromatography followed by derivatization with ninhydrin and detection at 570 nm and 440 nm. The amino acids were qualified and quantified by the amino acid standard solution (Membrapure) and expressed as mg/g.

Protein digestibility was determined in vitro by digesting the freeze-dried sample under simulated conditions of oral, gastric and intestinal phases, according to the procedure of INFOGEST method [15]. The digested sample was centrifuged at 500 ×g for 20 min at 4°C. The supernatant was collected for protein content measurement by Kjeldahl method (AOAC 950.48) [16]. The in vitro digestibility of protein was calculated as the percentage of protein content in digested sample and total protein content. For comparison, soy protein isolate was used as the benchmark.

3.2 Structural properties

The molecular weight (MW) distribution of proteins in hydrolyzed and non-hydrolyzed samples was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [17]. Protein samples were mixed with Laemmli buffer and 5% w/v β-mercaptoethanol, heated at 95°C for 5 min, then cooled on ice bath. Samples were loaded onto 4–20% w/v precast polyacrylamide gels (Bio-Rad Laboratories, Hercules, California, USA) and ran at 200 V for 35 min using Tris-tricine-SDS buffer pH 8.3. Gels were stained with Bio-Safe Coomassie and destained with deionized water. Protein bands were identified by comparison with protein markers (2–250 kDa), and analyzed using an image analyzer (Fusion FX7, Vilber, Collégien, France).

The secondary structure was analyzed using FTIR spectroscopy (Nicolet iS50, Thermo Fisher Scientific, Waltham, Massachusetts, USA) in attenuated total reflectance mode. Spectra were collected from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1}, averaging 64 scans, follwing the previously described method [18, 19]. Spectral deconvolution and second-derivative analysis were performed using Omnic software v6.2 (Thermo Fisher Scientific). The amide I region was used to identify secondary structures with characteristic bands at 1654–1658 cm^{-1} for α-helix, 1624–1642 and 1691–1696 cm^{-1} for β-sheet, 1666–1688 cm^{-1} for β-turn, and 1646–1650 cm^{-1} for random coil [20].

3.3 Functional properties

Protein solubility was evaluated across pH 3, 5, and 7 using 0.2 M phosphate-citrate buffers. Chickpea meal hydrolysates were dispersed (3 mg protein/mL), stirred for 30 min, and centrifuged at 4000 ×g for 15 min at 10°C. Soluble protein in the supernatant was quantified by the Lowry assay [21]. Protein solubility (%) was calculated as the percentage of protein concentration in the

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supernatant and the total protein concentration in the dispersion [22].

Heat stability of CPM and CPHs were determined according to the method modified from that of Crowley and Dowling [23]. The protein solution (3 mg protein/mL in 0.2 M phosphate-citrate buffer pH 7) was heated in a 95ºC water bath for 30 min and immediately cooled down to room temperature in an ice bath. The dispersions were centrifuged at 4000 ×g for 30 min to remove the protein curd and the protein content in the supernatant was analyzed by Lowry's assay as described earlier. Heat stability was determined as the percentage of protein content in the supernatant after heating and that of the unheated solution.

3.4 Bioactive properties

Antioxidant activities were determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) [24], ferric reducing antioxidant power (FRAP) [25], and oxygen radical absorbance capacity (ORAC) [26] assays. DPPH, FRAP and ORAC values were determined against the standard curves of Trolox solution, and expressed as Trolox Equivalent (µmol TE/g protein). Anti-hypertensive activity was assessed by angiotensin-converting enzyme (ACE) inhibition using a modified method described elsewhere [27].

Anti-inflammatory activity was evaluated by measuring nitric oxide (NO) production and interleukin-6 (IL-6) secretion in RAW 264.7 cells. Cell viability was assessed using the MTT assay, while IL-6 levels were determined using an ELISA kit (R&D Systems, Minneapolis, Minnesota, USA), following treatment with or without 50 ng/mL lipopolysaccharide (LPS). Absorbance was measured at 450 nm using a microplate reader, and cytokine concentrations were calculated from standard curves. Results were expressed as percentage of cell viability and percentage of inhibition for NO and IL-6, as described by Baek et al [28]. Cell viability (%) = (A / B) × 100 and Inhibition (%) = [1 – (A / B)] × 100, where A is the sample and B is the untreated or LPS-induced control.

4. Statistical analyses

All experiments were performed in triplicate, and results were expressed as means ± standard deviations. Statistical analysis was conducted using one-way ANOVA, and differences among means were evaluated by Tukey's HSD test at p-level of 0.05 using statistical software (SPSS Version 29, IBM, Armonk, New York, USA).

RESULTS AND DISCUSSION

1. Nutritional properties

CPM contained 17.88% protein on a dry basis (Table 1), which is slightly lower than that reported elsewhere [29]. The hydrolyzed meals, CPH-AP and CPH-NP, had higher protein content, suggesting that hydrolysis by protease could liberate protein from the meal, especially at alkali condition of hydrolysis by AP. Ash content in both hydrolysates was higher than the native CPM, while fat content was lower. The higher ash content of the hydrolysates was probably due to the accumulation of inorganic salts formed during acid–base pH adjustment, the presence of mineral ions introduced through enzyme formulations or buffer components, and the proportional increase in

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ash content resulting from the reduction of organic matter during protein hydrolysis. Similar increase in ash content of protein hydrolysates has also been reported elsewhere [30, 31].

Table 1 Proximate composition, amino acid composition and *in vitro* protein digestibility of chickpea meal and its hydrolysates prepared using alkaline protease and neutral protease

Composition	Content[†]			Recommended pattern[§]
	CPM	CPH-AP	CPH-NP	
Proximate (% dry basis)				
Protein (Nx6.25)	17.88 ± 5.31^{C}	32.54 ± 0.53^{A}	28.82 ± 0.38^{B}	–
Fat	5.30 ± 1.33^{A}	0.63 ± 0.01^{B}	0.47 ± 0.01^{C}	–
Ash	1.41 ± 0.17^{C}	12.51 ± 0.20^{B}	13.60 ± 0.18^{A}	–
Carbohydrate	75.40 ± 6.63^{A}	53.18 ± 0.87^{B}	56.18 ± 0.75^{B}	–
Amino acid (mg/g protein)				
Essential amino acids^{ns}	370.37 ± 1.95	364.70 ± 3.50	363.70 ± 6.12	
Cysteine	8.53 ± 0.32^{A}	6.33 ± 0.22^{B}	10.02 ± 2.45^{A}	22
Methionine	3.48 ± 0.70^{B}	2.13 ± 0.11^{C}	4.08 ± 0.21^{A}	
Histidine^{ns}	28.57 ± 1.05	27.75 ± 0.62	27.73 ± 2.19	15
Isoleucine^{ns}	40.15 ± 0.73	41.83 ± 0.74	26.29 ± 18.75	30
Leucine^{ns}	76.99 ± 2.58	73.26 ± 0.42	74.90 ± 3.62	59
Lysine^{ns}	68.83 ± 3.03	72.06±0.67	75.24 ± 3.28	45
Phenylalanine^{ns}	60.75 ± 0.08	57.62±2.01	61.49 ± 3.87	30
Threonine^{ns}	25.74 ± 1.27	22.18 ± 0.44	19.74 ± 6.32	23
Tryptophan	12.28 ± 3.63^{A}	7.08 ± 0.12^{B}	13.15 ± 0.78^{A}	6
Valine	45.06 ± 0.12^{B}	54.47 ± 0.40^{A}	51.06 ± 2.56^{A}	39
Non-essential amino acids^{ns}	629.63 ± 1.95	635.30 ± 3.50	636.30 ± 6.12	
Alanine	44.64 ± 1.51^{B}	48.32 ± 0.20^{A}	54.08 ± 13.47^{AB}	–
Arginine	101.15 ± 7.42^{A}	91.15 ± 0.53^{B}	94.71 ± 2.69^{AB}	–
Aspartic acid^{ns}	122.69 ± 2.96	127.23 ± 1.43	126.13 ± 3.30	–
Glutamic acid	216.20 ± 3.33^{B}	234.16 ± 1.31^{A}	219.02 ± 13.22^{AB}	–
Glycine	41.25 ± 0.13^{B}	44.42 ± 0.07^{A}	47.37 ± 3.82^{AB}	–
Proline	35.58 ± 2.96^{B}	44.34 ± 4.70^{A}	44.14 ± 1.55^{A}	–
Serine	45.56 ± 1.45^{A}	24.48 ± 0.31^{C}	31.51 ± 2.94^{B}	–
Tyrosine^{ns}	22.56 ± 2.72	21.19 ± 1.05	19.35 ± 3.03	–
In vitro protein digestibility (%)^{ns}	96.51 ± 3.36	100.21 ± 0.76	98.66 ± 3.58	–

[†]Means ± standard deviations of 3 replicates; [§]Recommended pattern for adults; [ns]Mean values within a row are not significantly different ($p>0.05$); [A,B,C]Means within a row with different superscript letters are significantly different ($p<0.05$).

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The amino acid composition of CPM (Table 1) reveals its total essential amino acid content of 370.37 mg/g protein. Glutamic acid is the most abundant amino acid, followed by aspartic acid and arginine, while sulfur-containing amino acids, namely cysteine and methionine, are the limiting amino acids which is common for chickpea and other peas [32]. Compared with the recommended values, most essential amino acids met or exceeded the recommended pattern for adults aged >18 years, except sulfur-containing amino acids and threonine [33]. A previous study reported that the total essential amino acid content in chickpea protein isolate was 355.7 ± 5.30 mg/g protein [34], which about similar to that of our study. Amino acid profile of both hydrolysates was indifferent from CPM, though slight changes were observed in content of some amino acids. Interestingly, CPH-AP had the least amount of cysteine and methionine among its counterparts. *In vitro* protein digestibility of CPH-AP and CPH-NP was >98%, which was slightly higher than that of CPM but significantly greater than that of soy protein isolate (91.70 ± 0.36%) analyzed in this study. This indicates that the partial hydrolysis did not adversely affect the quality of protein in CPM.

2. Protein structure

The MW distribution pattern obtained from SDS-PAGE (Figure 1) reveals differences in MW profiles of CPM, CPH-AP, and CPH-NP due to enzymatic hydrolysis. CPM had a largest MW of 120.1 kDa, with major bands at 75, 50, 25–37, and 20 kDa. Enzymatic hydrolysis shifted the MW distribution of both hydrolysates to smaller peptides. For CPH-AP, the largest MW decreased to 58.8 kDa, with major bands at 25–37 and 20 kDa, indicating extensive protein breakdown. CPH-NP showed a largest MW of 97.4 kDa and bands at 50, 25–37, and 20 kDa. In addition, the band of MW <5 kDa was observed in both hydrolysate samples, while it was absent in CPM. These findings showed that AP had greater efficacy than NP in reducing MW of CPM, which is well consistent with the higher DH of CPH-AP.

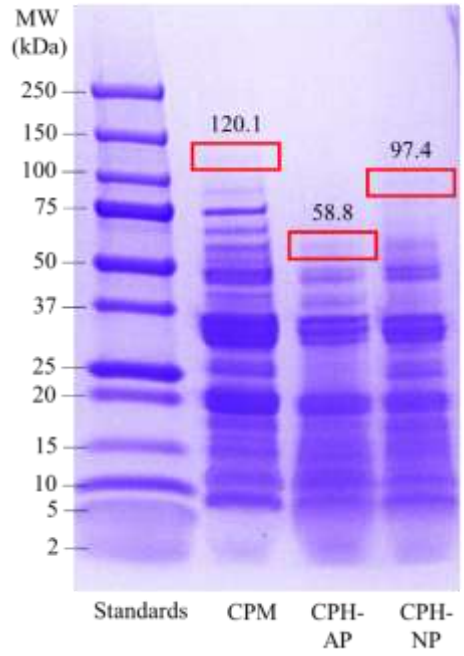

Figure 1 Typical molecular weight distribution of proteins of chickpea meal (CPM) and its hydrolysates prepared using alkaline protease (CPH-AP) and neutral protease (CPH-NP). Squares indicate the largest molecular weight fraction of each sample.

The FTIR spectra of CPM, CPH-AP, and CPH-NP are compiled in Figure 2. Similarly for all samples, peaks with greater intensity were observed at around 3300 cm^{-1} (amide A), 2930 cm^{-1} (C—H stretching), 1630 cm^{-1} (amide I, C=O

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stretching) 1540 cm^{-1} (amide II, N—H bending and C—N stretching), 1230 cm^{-1} (amide III, C—N stretching and N—H deformation) [35]. The amide I band in the FTIR spectrum (1600–1700 cm^{-1}) was deconvoluted to determine the protein secondary structure, as its frequency is sensitive to structural elements due to hydrogen-bonding environments. Different secondary structure have different amide I vibrational frequencies, i.e., 1650–1658 cm^{-1} for α-helix, 1610–1640 cm^{-1} for β-sheet, 1660–1700 cm^{-1} for β-turn, and 1640–1650 cm^{-1} for random coil [36]. As shown in Table 2, CPM contained a higher proportion of random coil and β-turn, indicating a flexible structure. In contrast, enzymatic hydrolysis shifted the structure of CPH-AP and CPH-NP toward more ordered conformations, with increased α-helix and β-sheet contents, suggesting enhanced compactness and stability. The sum of α-helix and β-sheet structures represents the total intermolecular hydrogen bonds, which reflects the degree of protein compactness [37]. The higher combined content of α-helix and β-sheet in CPH-AP and CPH-NP compared to CPM aligns with findings by Ceylan et al [38]. The similar increase in α-helix after Alcalase treatment of potato protein isolate have been previously reported by Akbari et al [39].

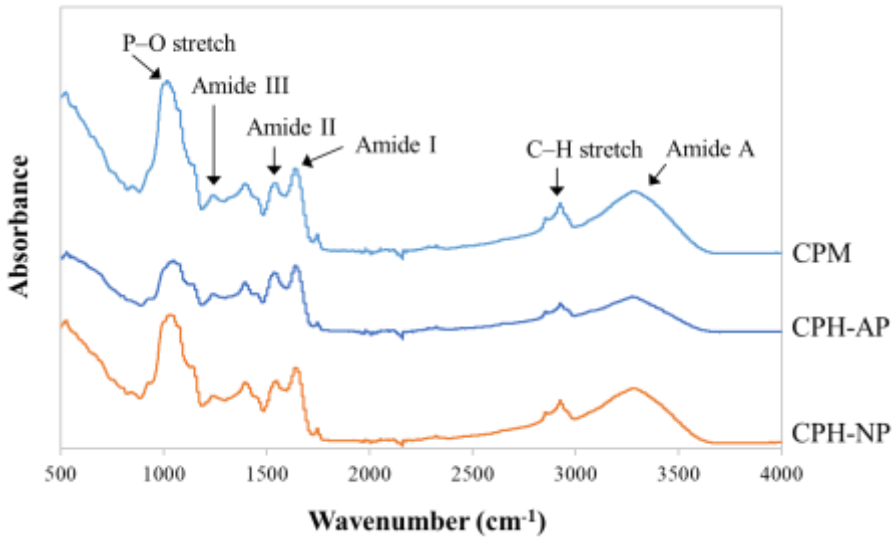

Figure 2 Typical FTIR spectra of chickpea meal (CPM) and its hydrolysates prepared using alkaline protease (CPH-AP) and neutral protease (CPH-NP). Arrows indicate peaks of greater intensity.

However, the increase in β-sheet structures observed in this study contrasts with Ceylan et al [35] but supports findings by Li et al [40], who reported an increase in β-sheet formation due to hydrophobic aggregation in rice proteins. In addition, Carbonaro et al [41] also noted increased β-sheet content from intermolecular aggregation in thermally-treated glycinin from 11S soybean protein. Conversely, structural transitions toward random coil have been associated with looser conformations [42]. In this study, the observed decrease in random

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coil and increase in ordered structures (α-helix, β-sheet, β-turn) in both hydrolysates may result from the partial hydrolysis, as evidenced by their DH and MW (Figure 1). It is plausible that hydrolysis, either by AP or NP, only cleaved the globular protein aggregates in CPM into smaller globules. In turn, there were more exposed globular structures, leading to increases in α-helix, β-sheet, and β-turn.

Table 2 Secondary structure composition of chickpea meal and its hydrolysates prepared using alkaline protease and neutral protease

Samples	Secondary structure composition (%)†			
	α-helix	β-sheet	β-turn	Random coil
CPM	13.45 ± 5.44[a]	10.40 ± 3.78[b]	31.29 ± 3.27[a]	44.86 ± 5.96[a]
CPH-AP	29.31 ± 16.10[a]	28.85 ± 4.49[a]	12.03 ± 4.17[b]	29.81 ± 7.44[ab]
CPH-NP	31.12 ± 9.13[a]	30.27 ± 11.17[a]	18.64 ± 12.23[ab]	19.96 ± 8.07[b]

†Means ± standard deviations of 3 replicates; [a,b,c]Means within a column with different superscript letters are significantly different ($p<0.05$).

3. Functional properties

The protein solubility of CPM, CPH-AP, and CPH-NP across different pH levels is shown in Table 3. Protein solubility of all samples significantly increased ($p < 0.05$) with the increasing pH, particularly from pH 5 to pH 7. Liu et al [37] also reported that plant proteins, including soy protein isolate, *Cyperus esculentus* protein, and flaxseed protein, exhibited the lowest solubility at pH 3 due to their isoelectric points. Among the three samples, CPM exhibited the lowest protein solubility, with a maximum of only 33.48 ± 7.35%. Limited enzymatic hydrolysis markedly enhanced solubility at all pH levels, particularly for CPH-AP, which reached 97.36 ± 6.86% at pH 7. Notably, CPH-AP and CPH-NP increased the solubility of the resulting hydrolysates by approximately 3–5 times and 2–3 times, respectively, compared to CPM.

Heat stability was determined after the samples at pH 7 were subjected to heating at 95°C for 30 min, which was close to that used in commercial sterilization process. It was found that heat stability of CPM was 64%; while CPH-AP and CPH-NP were more heat stable that they almost did not coagulate with heat (Table 3). Stability of the hydrolysates was closed to 100%. The improved heat stability of the hydrolysates was due to their smaller MW and higher solubility. This suggested that the hydrolysate of CPM can be added to products without causing coagulation and precipitation during further heat treatment.

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Table 3 Protein solubility at different pH values, and heat stability of CPM, CPH-AP, and CPH-NP

Samples	Protein solubility (%)†			Heat stability at
	pH 3	pH 5	pH 7	pH 7 (%)†
CPM	7.86 ± 3.31^{bC}	8.31 ± 2.18^{cC}	33.48 ± 7.35^{cA}	64.16 ± 0.43^{b}
CPH-AP	37.22 ± 8.75^{aC}	51.79 ± 5.79^{aB}	97.36 ± 6.86^{aA}	99.19 ±6 .12^{a}
CPH-NP	17.37 ± 9.43^{bB}	21.38 ± 5.91^{bB}	78.88 ± 5.22^{bA}	95.88 ± 3.32^{a}

†Means ± standard deviations of 3 replicates; ^{a,b,c}Means within a column with different superscript letters are significantly different ($p<0.05$); ^{A,B,C}Means of protein solubility within a row with different superscript letters are significantly different ($p<0.05$).

4. Bioactive properties

4.1 Antioxidant properties

The antioxidant activities of CPM, CPH-AP, and CPH-NP, assessed by DPPH, FRAP, and ORAC assays, are shown in Table 4. None of the samples demonstrated activity in the DPPH assay, which evaluates radical scavenging via both hydrogen atom transfer (HAT) and single electron transfer (SET) mechanisms [43]. This suggests a limited capacity of the samples to scavenge free radicals. DPPH reaction mechanisms are known to be altered by many environmental factors, including water and solvents, pH, oxygen, and light exposure, as well as the steric inaccessibility of the DPPH radical site, especially in mixtures of compounds [44]. It is likely that these phenomena could depress the DPPH assay. Similar low DPPH activity was previously reported in lentil and black soybean protein hydrolysates [45]. FRAP values varied among samples, with CPH-NP exhibiting the highest value (23.68 ± 1.20 µmol TE/g protein), followed by CPH-AP (21.14 ± 1.73 µmol TE/g protein) and CPM (15.32 ± 0.47 µmol TE/g protein). FRAP assesses antioxidant capacity via the SET mechanism by measuring the reduction of Fe^{3+} to Fe^{2+} [43]. The elevated FRAP values in CPH-AP and CPH-NP suggest the improved electron-donating abilities compared to CPM. A previous study [46] reported that the FRAP content of chickpea protein hydrolysates treated with Bioprotease was approximately 23 µmol TE/g protein, which is consistent with the findings of this study.

ORAC values showed a notable increase in CPH-AP (924.34 ± 150.60 µmol TE/g protein), nearly double that of CPM (449.69 ± 54.14 µmol TE/g protein) and CPH-NP (534.60 ± 49.06 µmol TE/g protein). ORAC evaluates antioxidant activity through the HAT mechanism by measuring the capacity to neutralize peroxyl radicals [43]. Our findings demonstrate notable improvements in antioxidant activity following enzymatic hydrolysis, particularly in FRAP and ORAC values. The enhanced FRAP and ORAC values, especially of CPH-AP, may result from the release of smaller antioxidative peptides during hydrolysis [47]. Antioxidative properties are largely associated with hydroxyl groups present in the structure of bioactive peptides [48].

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Table 4 Antioxidant properties of chickpea meal and its hydrolysates prepared using alkaline protease and neutral protease

Samples	DPPH (μmol TE/g protein)†	FRAP (μmol TE/g protein)†	ORAC (μmol TE/g protein)†	ACE inhibitory (%)†, ‡
CPM	ND	15.32 ± 0.47^{c}	449.69 ± 54.14^{b}	84.26 ± 3.85^{b}
CPH-AP	ND	21.14 ± 1.73^{b}	924.34 ± 150.60^{a}	94.74 ± 0.62^{a}
CPH-NP	ND	23.68 ± 1.20^{a}	534.60 ± 49.06^{b}	92.82 ± 1.44^{a}

ND: Not detected; †Means ± standard deviations of 3 replicates; ‡3 mg protein/mL assay; ^{a,b,c}Means within a column with different superscript letters are significantly different ($p<0.05$).

4.2 Anti-hypertensive properties

Activity on inhibition of angiotensin converting enzyme (ACE), which is an enzyme that controls blood pressure, was adopted for the determination of anti-hypertensive properties of CPM and its hydrolysates (Table 5). ACE plays a key role in blood pressure regulation by converting angiotensin I to the vasoconstrictor angiotensin II [49]. Among the samples, CPH-AP demonstrated the highest ACE inhibitory activity (94.74 ± 0.62%), followed closely by CPH-NP (92.82 ± 1.44%), while CPM showed significantly lower activity (84.26 ± 3.85%). The predominance of hydrophobic amino acids in chickpea meal may contribute to the ACE-inhibitory activities of CPM, CPH-AP, and CPH-NP, as hydrophobic residues are known to enhance ACE inhibition [50]. The greater inhibitory activity observed in the hydrolysates suggests that enzymatic hydrolysis promoted the release of bioactive peptides, potentially enhancing their antihypertensive efficacy [51]. This is consistent with a previous report [52] that chickpea protein hydrolysates effectively reduced systolic blood pressure in hypertensive rats, with higher DH correlating with increased bioactivity. Lower MW peptides are also commonly linked to stronger ACE inhibition and better bioavailability. So, the fact that CPH-AP had the highest DH, yet lowest MW (Figure 1) might be responsible for its highest ACE inhibitory activity among the three samples. Furthermore, the structural modifications in secondary structures induced by enzymatic hydrolysis (Table 2) could also impart in the improved the inhibitory effect against ACE of the hydrolysates.

4.3 Anti-inflammatory properties

The anti-inflammatory properties of CPM, CPH-AP, and CPH-NP were determined as cell viability and inhibition of NO and IL-6 secretion and are presented in Table 5. All samples showed high MTT cell viability (>95%), confirming their non-cytotoxicity. CPH-AP demonstrated strong inhibition of NO (73.39 ± 5.53%) and IL-6 (66.02 ± 4.35%), suggesting that hydrolysis with AP released bioactive peptides capable of modulating inflammatory pathways [53]. In contrast, CPH-NP showed moderate inhibition of NO (13.06 ± 2.17%) and IL-6 (16.49 ± 3.13%), indicating that hydrolysis with NP could not be effective in generating peptides with anti-inflammatory properties as did AP.

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CPM, in its native form, exhibited barely activity on anti-inflammation, with NO and IL-6 inhibition of 5.1 ± 3.80% and 8.66 ± 3.45%, respectively. This underscores the limited functionality of the intact protein in modulating inflammatory responses. Anti-inflammatory effects have also been reported in various plant protein hydrolysates. Rivera-Jiménez et al [54] demonstrated that peptides from trypsin-hydrolyzed rice protein significantly reduced NO production and inhibited the expression of inducible nitric oxide synthase and pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β.

Table 5 Anti-inflammatory properties of chickpea meal and its hydrolysates prepared using alkaline protease and neutral protease

Samples	MTT cell viability (%)$^{\dagger, ns}$	NO inhibitory (%)†	IL-6 inhibitory (%)†
CPM	95.11 ± 3.87	5.1 ± 3.80^{c}	8.66 ± 3.45^{c}
CPH-AP	96.39 ± 4.49	73.39 ± 5.53^{a}	66.02 ± 4.35^{a}
CPH-NP	96.03 ± 3.75	13.06 ± 2.17^{b}	16.49 ± 3.13^{b}

†Means ± standard deviations of 3 replicates; ^{ns}Mean values within a column are not significantly different ($p>0.05$); ^{a,b,c}Means within a column with different superscript letters are significantly different ($p<0.05$).

CONCLUSIONS

This study highlights the valorization potential of chickpea meal, a currently underutilized by-product of chickpea milk, into high-value functional ingredients with nutritional benefits. Through limited enzymatic hydrolysis using commercial proteases, significant improvements were observed in protein structure, protein solubility, heat stability, antioxidant capacity, anti-hypertensive and anti-inflammatory activities, while maintaining the nutritional quality of chickpea protein. However, the specific peptides were not yet identified, which restricted a complete understanding of responsible peptides for the observed biological effects. Future research should focus on a more in-depth characterization and sequencing of the purified fractions of the hydrolysates for a better understanding of their functional and bioactive properties, as well as *in vivo* and clinical evaluations.

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