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Assessment of the Physicochemical, Antioxidant, *in-vitro* Anti-diabetic and Nutritional Characteristics of Pigeon Pea Protein Hydrolysates

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Abstract

Background and Objective: *Cajanus cajan* (pigeon pea) seeds include special characteristics that can serve as alternative vegan protein sources. The aim of this study was to investigate bioactive peptides in the pigeon pea using economically feasible method of acid and enzymatic hydrolysis.

Material and Methods: In this study, pigeon pea was subjected to hydrolysis by two methods of acid and enzymatic hydrolysis. The generated hydrolysates were characterized by result analysis of the protein content and yield, degree of hydrolysis, anti-nutritional profile, Fourier transform infrared spectroscopy, antioxidant assay of 2,2-diphenyl-1-picryl hydrazyl, hydroxyl radical scavenging assay, metal chelating ion assay and reducing power. Moreover, antidiabetic effects were assessed using α-amylase inhibition assay.

Results and Conclusion: Pigeon pea was digested by acid (pH 4) and enzyme hydrolysis, further subjected to membrane filtration to achieve peptide fractions with bioactive characteristics. The hydrolyzed pigeon pea showed good increased protein contents and degree of hydrolysis, compared with the control. Degree of hydrolysis were 62.7% for acid, 68.42% for enzyme and 34.32% for unhydrolyzed proportion. Hydrolyzed samples included Fourier transform infrared peaks at 3500–4000 cm^{-1}, showing amides I and II. The resulting peptides after the hydrolysis showed a higher range in acid hydrolysis (250–20 kDa), whereas the EH fractions showed a very low molecular weight of less than 15 kDa. Peptides produced by AH demonstrated considerable bioactive characteristics, compared to EH antioxidant and anti-diabetic characteristics against the standards. This study highlights production of pigeon pea protein hydrolysates using two methods of traditional (acid) and modern (enzymatic), showing that acid hydrolysate can be a cheap economical method for generating protein hydrolysate with good bioactive characteristics.

Conflict of interest: The authors declare no conflict of interest.

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1. Introduction

Recently, high demand for plant proteins have been reported due to production of plant-based meat, shifting consumer preferences and increased awareness of their role in health and fitness. Legume seeds include a critical place in the human diet worldwide as a rich source of proteins. Legumes are classically called the poor man's meat when animal proteins are limited or when poverty, spiritual or holy preferences prevent consumption of meat. One of the most essential dietary legumes is *Cajanus cajan*, commonly known as pigeon pea and red gram in English [1, 2]. Production statistics of pigeon pea reveal that India contributes to nearly 90% of the global production. Despite having a high nutritional profile, pigeon pea is underused and received little attention from research and development to unlock its potential uses in food industries. *Cajanus cajan* is a significant source of proteins, vitamins and minerals rich in essential amino acids (EAA), with large quantities of lysine, which is often a limiting factor in plant-based proteins within the dietary legumes playing critical roles in human nutrition. Numerous studies on pigeon pea have revealed significant findings such as antioxidant, anti-hypertensive, anti-diabetic, anti-carcinogenic, anti-coagulant, anti-inflammatory and certain satiety effects. It is the most appropriate alternative for individuals with

allergies or sensitivities to other popular sources such as soy, dairy, or wheat. It offers a hypoallergenic option for incorporating proteins into various food systems. Pulse proteins are a rich source of potential bioactive sites with the help of hydrolysis, autolysis, gastrointestinal digestion and fermentation. Complex protein in pigeon pea when subjected to artificial hydrolysis, natural gastrointestinal digestion or fermentation hydrolysis forms peptides with good bioactive characteristics that can be used as functional foods, benefiting human health [1].

Multiple processes have been used for deriving protein hydrolysates, chemical and modified, of which, enzymatic protein hydrolysis is the most common process. Currently, most proteins are hydrolysed using proteolytic enzymes at the ideal temperature and pH. These often target particular peptide bonds, resulting in the release of AAs and peptides of various sizes [3]. In chemical methods, acid/alkaline hydrolysis are a conventional method. In chemical method, it is seen that mostly non-essential amino acids (asparagine, glutamine, cysteine, and tryptophan) are destroyed that are difficult to recover by acidic hydrolysis; hence, neutralization with a base (hydroxide) is recommended after heating. The hydrolysed protein is further subjected to membrane filtration or purification method [4]. The major problem of enzymatic hydrolysis is its expensive costs as well as presence of enzyme inhibitors in raw materials. The need of careful optimization and handling is essential, which can lead to enzyme denaturation or inactivation, resulting in incomplete hydrolysis with lower yields. An alternate economical method that can be used for protein hydrolysis includes acid hydrolysis. Studies have shown that essential amino acids (EAAs) such as aspartic acid, glutamic acid, proline, glycine, alanine, leucine, phenylalanine, histidine, and arginine can be achieved by acid hydrolysis (AH) [4]. Therefore, the aim of this study was to investigate the best cheap method for the production of pigeon pea protein hydrolysates via AH or enzymatic hydrolysis (EH) with good bioactive characteristics.

Bioactive peptides are addressed as nutraceuticals with health advantages associated with illness prevention or therapy. Studies on the antioxidant characteristics of crude protein hydrolysates have been carried out by several researchers [5–8]. Nutritional characteristics of pigeon pea have been associated with decrease in occurrence of various cancers, HDL cholesterol, type-2 diabetes, and heart diseases. These include compounds such as protease inhibitors, non-antinutritional components and angiotensin I-converting enzyme (ACE) inhibitor with possible beneficial characteristics [5,9,10]. Studies have shown that foods packed with antioxidants provide functional health benefits by acting as exogenous sources of antioxidants to neutralize oxidants. Recently, foods rich in protein-derived peptides, typically achieved through the hydrolysis of food proteins, have been assessed for potential therapeutic functions in preventing cellular damages from oxidative stress that promotes human health. These pulse proteins are the most investigated naturally occurring alternatives to synthetic antioxidants and antidiabetic characteristics. Pulse proteins have become popular due to their availability, accessibility, affordability and simpler derivative [11]. Furthermore, the novelty of this study is to highlight advantages, disadvantages and cost-effectiveness of various methods for producing superior bioactive peptides from pigeon pea. Research on the specific effects of AH on pulse hydrolysates is in its early stages, however presenting an exciting opportunity to tailor their functional characteristics.

2. Materials and Methods

Unpolished pigeon pea seeds were purchased from a local market in Karnataka, India. Olive oil was purchased from a local supermarket in Vishakhapatnam, India. All the chemicals and reagents were in analytical grade from Sigma, Germany, Merck, Germany, and Himedia, India, and provided by a local vendor.

2.1. Preparation of the protein hydrolysate

Sample preparation was carried out based on a method described previously [12]. Seeds purchased were washed, sun-dried and powdered into fine meal using 40-mesh sieve. Roasted seeds were subjected to fat extraction process for nearly 6 h using an ethanol extraction-based system. Mixture was further subjected to distillation to achieve the final fat-free product for hydrolysis. For acid hydrolysis, hydrolysates were prepared using 6 M of HCl and were then added to defatted pigeon pea flour. Extracted samples were with 6 M hydrochloric acid per mL for various time constraints under high pressure at 121 PSI for 3–4 h. Precipitated protein at isoelectric pH was removed from the suspension by centrifugation at 12298 g for nearly 20–30 min, adjusted to pH 7.0 with 0.1 M NaOH, lyophilized and stored at 4 °C [13]. For EH, pigeon pea sample was hydrolysed enzymatically using protease pigeon pea and fat-free suspensions were prepared using 100 g of the samples that was brought to a volume of 1 L with 0.10 M phosphate buffer (pH 7–7.4 under optimum conditions with the enzyme). The enzyme complex was added for each of the experiments at room temperature (RT), based on the enzyme/substrate (E/S) ratios (m/m) (0.1, 0.3, 0.5, 0.7, and 1). The reaction mixture was transferred into a bioreactor with a capacity of 20 L. Ranges used to assess variables of pH, temperature, and time included 1–9, 50–70 °C, and 100–180 min, respectively. Separation of the solid from the supernatant was carried out for each sample when the enzymatic activity ended via heating at 90 °C for 20 min. After decanting the samples, supernatant was centrifuged at 2490 g. Further selective filtration was carried out using 0.4-

μm membrane filtration and the supernatant was freeze-dried and stored at 4 °C until further use [10,11,14].

2.2 Proximate composition of pigeon pea

Pigeon pea seeds (unhydrolysed, defatted, and hydrolysate) were analyzed for crude fiber, ash, and relative humidity using standardized methods by the Association of Official Analytical Chemists (AOAC) [15]. Crude fat was assessed using Soxhlet solvent extraction system [13]. Crude fat was calculated using the Eq. 1:

$$Crude\ fat\ (\%) = \frac{W_2 - W_1}{P} \quad \text{Eq. 1}$$

Where, W_1 was the empty thimble, W_2 was weight of the sample and thimble and P was weight of the sample.

2.3. Protein content assessment

Kjeldahl protocol was used as described previously [16]. Briefly, 200 mg of the samples were used in the analysis. Kjeldahl method was used to assess the protein content based on the protocol of AOAC IS 7219 [15]. The assessed nitrogen content was multiplied by 6.25 (the nitrogen-to-protein conversion factor for legumes) to achieve the final protein concentration of the samples [13] using Eq. 2:

$$Nitrogen\ content\ (N)\ (g) = (a - 2b) - (c - 0.2d) \times 0.007 \quad \text{Eq. 2}$$

Where, a was the volume in 0.5 N acid assessed for distillation, b was the volume in 0.1 N base used for back titrating a, c was the volume in 0.5 N acid used for blank distillation a, and d was the volume in 0.1 N alkali used for back titrating c.

2.4. Degree of hydrolysis

The O-phthalaldehyde assay (OPA) procedure was used for assessing degree of hydrolysis (DH) as described by Nielsen [13]. The OPA was a sensitive technique widely used for pulse proteins [14] and DH was calculated using Eq. 3:

$$(Serine - NH_2) = \frac{(\text{Sample OD} - \text{Blank OD})}{(\text{Standard OD} - \text{Blank OD})} \times 0.9516 \frac{meqv}{L} \times V \times \frac{100}{X} \times P \quad \text{Eq. 3}$$

Where, V was the sample volume (0.1 lL), X was the sample weight (0.125 g), P was the soluble protein content (90%) of the sample and serine-NH_2 was in meqv serine-$NH_2 \cdot g^{-1}$ protein (Eq. 4).

$$h = \frac{\text{Serine} - \text{NH}_2 - \beta}{\alpha\ \text{meqv/protein}} \quad \text{Eq. 4}$$

Where, α and β were respectively the constants of 1.00 and 0.40 for the raw materials that were not assessed. The DH was calculated using Eq. 5

$$DH\ (\%) = \frac{h}{h_{tot}} \times 100 \quad \text{Eq. 5}$$

Where, h_{tot} was the total number of peptide bonds per protein equivalent.

2.5. Protein yield assessment

The yield of protein pigeon pea samples (defatted, AH, and EH) was assessed as per modifications and was calculated using Eq. 6:

$$Yield\ (g \cdot 100\ ml^{-1}) = \frac{\text{Weight of protein hydrolysate powder(g)}}{\text{Weight of raw material (100mL)}} \quad \text{Eq. 6}$$

2.6. Assessment of anti-nutritional factors

Anti-nutritional factors of tannic acid, total phenol content, phytic acid, and trypsin inhibition were carried out as previously described [15].

2.6.1. Total phenolic content assessment

The total phenol content of various samples was assessed quantitatively using Folin-Ciocalteu method; in which, gallic acid was taken as standard and the absorbance was measured at 760 nm [15].

2.6.2. Tannins assessment

Presence of tannins was assessed using Folin-Denis reagent spectrophotometric method at 700 nm [15].

2.6.3. Phytic acid content assessment

Phytic acid content of the samples was assessed spectrophotometrically at 480 nm, where Fe $(NO_3)_3$ was used as standard [15].

2.6.4. Trypsin inhibition assessment

Trypsin inhibition activity was assessed indirectly by inhibiting activity of the synthetic substrate [Nα-benzoyl-D,L-arginine p-nitroanilife hydrochloride (BAPNA)], which was subjected to hydrolysis by trypsin to produce yellow colored p-nitroanilide at 400 nm [17].

2.6.5. Saponin assessment

Saponins were assessed spectrophotometrically at 430 nm using saponin (0–40 μg) as standard [17].

2.7. Fourier transform infrared spectroscopy

The Fourier transform infrared (FTIR) spectroscopy spectra of pigeon pea AH were recorded using ALPHA-II FTIR spectrometer (Bruker Optics, Germany) fitted with an ATR (attenuated total reflectance) sampling device containing diamond crystals. The absorbance spectra were 4000–400 cm^{-1} (with a standard KBr beam splitter) at a spectral resolution of 4 cm^{-1} with 16 scans co-added and averaged. Additionally, 1–2 g of the sample were finely powdered for the scan. Data were translated into transmittance units [18].

2.8. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of unhydrolyzed and hydrolyzed samples was carried out using 5% stacking gel and 15% separating gel using Biobase gel system (Shandong, China) at 100 V. Gels were fixed in Coomassie brilliant blue (CBB) under RT and destained to visualize bands [18].

2.9. Amino acid composition

The pigeon pea fat-free unhydrolysed control and protein hydrolysates (AH and EH) were digested with 6 M of HCl for 24 h. Then, AA composition was assessed using HPLC 1260 Infinity Agilent system, USA, based on a method previously described [19]. Cysteine and methionine contents were assessed post performic acid oxidation as described previously [20] and the tryptophan content was assessed as described by Li et al. [8].

2.10. Assessment of functional characteristics

2.10.1. Water and oil absorption capacities

Water absorption capacities (WAC) of the protein was assessed [17, 18] and then calculated using Eq. 7:

$$WAC\ (g \cdot g^{-1}) = \frac{(V_1 - V_2)}{W} \quad \text{Eq. 7}$$

Where, W was the protein weight, V_1 was quantity of the distilled water (DW) and V_2 was volume of the water. For oil absorption capacities (OAC), 0.5 g of the samples were mixed with 5 mL of the appropriate vegetable oil and vortexed for 5 min. Slurry was centrifuged at 1968 g for 30 min and then weight of the adsorbed oil was assessed. The OAC was calculated using Eq. 8:

$$OAC\ (g \cdot g^{-1}) = \frac{(W_1 - W_2)}{W} \quad \text{Eq. 8}$$

Where, W was weight of the protein sample, W_1 was weight of the oil and W_2 was quantity of the free oil.

2.10.2. Emulsifying activity index (EAI)

Emulsifying ability of the samples (unhydrolyzed, defatted and hydrolyzed) was carried out based on a method with modifications [19]. The EAI was calculated using Eq. 9:

$$EAI\ (m^2 \cdot g^{-1}) = \frac{(2 \times 2.303 \times A_0)}{\varphi \times C} \quad \text{Eq. 9}$$

Where, A_0 was the absorption at 500 nm, φ was volume of the oil fraction, and C was protein concentartion of the sample.

2.10.3. Emulsifying stability index

For emulsifying stability index (ESI), samples at a protein concentration of 1% were mixed with 9 g of vegetable oil (e.g. olive oil) and then homogenized for 5 min using ultrasonic cell crusher noise isolating chamber [19]. Two aliquots were pipetted at 0 and 10 min and further diluted with 5 ml of 0.1% SDS solution. Absorbance of the solution was recorded at 500 nm (in min) and was calculated using Eq. 10:

$$ESI\ (min) = \frac{A_0 \cdot \Delta t}{A_0 - A_{10}} \quad \text{Eq. 10}$$

Where, A_0 was the absorbance at 0 min., A_{10} was the absorbance at 10 min, Δt was the time difference of 10 min.

2.10.4 Foaming characteristics

Foaming characteristics were assessed, including foaming capacity (FC) and foaming stability (FS). Briefly, 200 mL of the sample solutions (unhydrolysed, defatted, and hydrolysates) with 0.5% protein concentration at various ranges of pH (2–10) were homogenized for 10 min using ultrasonic cell crusher noise isolating chamber and φ 6 probes to induce air at RT [20]. Generally, FC and FS were calculated using Eqs. 11 and 12:

$$Foaming\ capacity\ (\%) = \frac{B}{A} \times 100 \quad \text{Eq. 11}$$

$$Foaming\ stability\ (\%) = \frac{C}{A} \times 100 \quad \text{Eq. 12}$$

Where, A was the volume before whipping (mL), B was the volume of foam at 0 min after whipping (mL) and C was the volume of foam at 20 min after whipping (mL).

2.11. Antioxidant characteristics

2.11.1 DPPH radical scavenging activity

For DPPH radical scavenging activity (DRSA), antioxidant activity was assessed using samples with a concentration of 1 mg·mL^{-1} that were mixed in water; 0.1 mg of DPPH-1:1 (v:v) was dissolved in 99.5% methanol [14]. Potential of the radical scavenging activity was assessed using absorbance at 517 nm and Shimadzu UV-1800, Nakagyo-Ku, Japan. In general, DPPH radical scavenging activity was calculated based on Eq. 13:

$$DPPH\ (\%) = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100 \quad \text{Eq. 13}$$

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

2.11.2. Hydroxyl radical scavenging activity

For hydroxyl radical scavenging activity (HRSA), nearly 100 µl of each sample with concentrations of 20–200 g were collected and mixed with 100 µl of ferrous sulphate (3 mM) and 100 µl of 1,10-phenanthroline (3 mM; dissolved in 0.1 M phosphate buffer; pH 7.4). Then, 100 µl of 0.01% hydrogen peroxide were added to the mixture to initiate the reaction. Mixture was incubated at 37 ºC for 1 h and absorbance was measured at 536 nm using Shimadzu UV-1800 spectrophotometer, Nakagyo-Ku, Kyoto, Japan [14]. Equation 14 was used to assess hydroxyl radical scavenging capacity.

$$HRSA\ (\%) = \frac{A_{sample} - A_{blank}}{A_{control} - A_{blank}} \times 100 \quad \text{Eq. 14}$$

Where, A_{sample} was absorbance of the sample, $A_{control}$ was absorbance of the control and A_{blank} was absorbance of the blank.

2.11.3. Metal ion-chelating assay

For metal ion-chelating assay (MCA), samples (100 µg) were mixed with 250 µl of 100 mM Na acetate buffer (pH 4.9) and 30 µl of FeC_{l2} (0.01%, w v^{-1}). Ferrozine (12.5 µl, 40 mM) was added to the mixture after incubation at RT for 30 min. Generally, EDTA was used as positive control. Binding of Fe (II) ions to ferrozine generated a colored complex that was measured at 562 nm using Shimadzu UV-1800, Nakagyo-Ku, Kyoto, Japan [14,21,22]. The ferrous ion chelating ability was calculated using Eq. 15:

$$Metal\ ion\ chelating\ ability\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad \text{Eq. 15}$$

Where, $A_{control}$ was absorbance of the control and A_{sample} was absorbance of the sample. Reduced glutathione (GSH) was assessed (1 mg·ml^{-1}) concurrently with the samples as positive control for all the antioxidant activity assays (DRSA, HRSA, and MCA).

2.11.4. Assessment of reducing power

Reducing power of the protein was assessed based on a modified method [8]. Protein hydrolysate solutions with various concentrations (1, 5, 10 and 15 mg·ml^{-1}) were prepared. Then, 1 ml of the vortexed sample was added to 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. Reaction mixture was rapidly vortexed and incubated at 50 °C for 20 min. Then, 2.5 ml of 10% TCA were added to the mixture and centrifuged at 12298 g for 10 min. Supernatant of 2.5 ml was mixed with 200 µl of deionized water and 40 µl of 0.1% $FeCl_3$. This was set to react for 10 min and absorbance was measured at 700 nm using Shimadzu UV-1800, Nakagyo-Ku, Japan.

2.12. Assessment of anti-diabetic characteristic using α-amylase inhibition activity

Inhibition of α-amylase activity was assessed using a protocol with slight modifications [14]. Briefly, 6 mM of NaCl and 100 µl of 20 ml l^{-1} sodium phosphate buffer (pH 6.9) were mixed with 100 µl aliquot of the sample containing 1 mg ml^{-1} of α-amylase solution. Further, 100 µl of 1% starch solution in 20 mM sodium phosphate buffer (pH 6.9, 6 mM) were mixed with the sample and incubated. Reaction was terminated by the addition of 200 µl of dinitrosalicylic acid and incubated at 100 °C for 5 min using boiling water bath. The reaction mixture was cooled down to RT, followed by the addition of 3 ml of double DW. Absorbance of the samples, Control 1 and Control 2 was measured at 540 nm. A standard synthetic drug (Glyciphage) was used to compare the values. Inhibition proportion of α-amylase activity was calculated using Eq. 16:

$$\propto -amylase\ inhibtion\ (\%) = \frac{Abs(control\ 2) - [Abs(sample) - Abs(control\ 1)]}{Abs\ \ (control\ 2)} \times 100 \quad \text{Eq. 16}$$

Where, Control 1 represented mixture of starch solution, protein sample excluding α-amylase enzyme and Control 2 represented mixture of starch solution, α-amylase enzyme excluding protein sample.

2.13. Statistical analysis

Analyses were carried out in three independent replications, with the outcomes subjected to one-way variance analysis. Statistically significant differences ($p \leq 0.05$) between the mean values were assessed using Tukey test and Origin Pro software v.8.1.

3. Results and Discussion

3.1. Proximate composition of pigeon pea

Crude fiber contents of unhydrolysed, defatted, acid, and enzyme hydrolysed pigeon pea seed samples were 7.56% ±0.96, 7.45% ±0.90, 7.10% ±0.80 and 6.95% ±0.80, respectively (Table 1). There was a little difference within the samples. The total ash contents were 3.76% ±0.46, 3.45% ±0.60, 3.97% ±0.70, and 4.05% ±0.60 for the highlighted samples, respectively.

Table 1. Characteristics of the chocolates used as semi-finished products

Nutritional composition (%)	UH	DF	AH	EH
Crude protein	20.07 ± 0.80[a]	21.51 ± 0.5[b]	22.05 ± 0.5[c]	24.05 ± 0.5[d]
Crude fat	4.45 ± 0.50[a]	2.78 ± 0.40[b]	2.18 ± 0.50[c]	1.95± 0.50[d]
Crude fiber	7.56 ± 0.96[a]	7.45 ± 0.90[a]	7.10 ± 0.92[a]	6.95 ± 0.80[b]
Crude ash	3.76 ± 0.46[a]	3.45 ± 0.60[b]	3.97 ± 0.70[c]	4.05 ± 0.60[d]
Dry matter	6.45 ± 0.15[a]	7.56 ± 0.10[b]	8.45 ± 0.15[c]	8.65 ± 0.15[d]
Moisture content	85.63 ± 0.50[a]	78.2 ± 0.50[b]	71.5 ± 0.50[c]	69.05 ±0.50[d]

For crude fat content, decreases in fat concentration were reported with a decreasing order from unhydrolysed to hydrolysate samples due to the extraction of defatted seeds using Soxhlet ethanol-based extraction, leading to the removal of fat in outer and inner pods of the seeds, which resulted in 4.45% ±0.50, 2.78% ±0.40, 2.18% ±0.50, and 1.95% ±0.50, respectively. Moisture contents of the samples were respectively 85.63% ±0.50, 78.2% ±0.50, 71.5% ±0.50, and 69% ±0.50 for unhydrolyzed, defatted and hydrolyzed samples [20].

3.2. Protein content

Protein content (PC) of the pigeon peas showed significant increase in hydrolysed seeds, compared to unhydrolysed (UH) seeds. Moreover, EH included a protein content of 24.05% ±0.5; AH included a protein value of 22.05% ±0.5 and UH included a protein content of 20.07% ±0.80. Significant increases in the total protein content of EH were recorded, compared with those of AH and UH (Table 2).

Table 2. Protein content (Dry matter) of unhydrolysed (UH), defatted (DF), enzymatic hydrolysis (EH), and acid hydrolysed pigeon pea (AH). *Different superscripts in the same column for one sample show the significant differences of $p \leq 0.05$.

Sample	Protein content (DM) (%)
UH	20.07 ± 0.8^{a}
DF	21.51 ± 0.5^{b}
AH	24.05 ± 0.5^{b}
EH	22.05 ± 0.5^{b}

Technically, standard Kjeldahl method measures total protein content on a dry weight basis. From the previously reported studies, it is understood that the protein content changes when subjected to hydrolysis; however, there are mild changes in the protein content, depending on the used methods. Values reported in the current study indicated that they were within a similar range of 21–28%. With the help of hydrolysis, germination and soaking in improved varieties, significant increases were reported in protein contents [17, 20]. The protein bioavailability increased due to the hydrolysis achieved in a shorter time. The hydrolysates solid-liquid extraction method was a further efficient economically acceptable process for producing fish protein hydrolysates. Due to increased DH, it could hydrolyze proteins in a shorter time than enzymatic hydrolysis could [2, 8].

3.3. Degree of hydrolysis

Table 3 shows that the enzymatically produced hydrolysates included 68.42% ±0.48, better than unhydrolysed samples with 34.32% ±0.57 DH when treated with acids [6, 23]. Increased rate of hydrolysis during the later stages of hydrolysis might be because of increase in cleaved peptides that were unfolded and exposed further to AH surface area, allowing for easier proteolytic cleavages. In this study, it was observed that the extractable protein affected degrees of hydrolysis, which was similarly observed in a study by Abbe et al., 2022 [2]. The DH for EH were detected as 68.42 ±0.48. Annihilation of the protein natural secondary structure and cleavage of peptide fragments from the larger UH protein structure increased the protein solubility, indicating sharp increases in DH. Additionally, increased hydrolysis might result in further hydrophobic AAs unfolding, which enhanced surface hydrophobicity. Increased surface hydrophobicity could help the formulation textural probiotic encapsulation [24]. The EH and AH included better DH and higher protein content but EH included defined limitations for its effects on peptide characteristics such as hydrolysate, length of peptide, enzyme-substrate ratio, specificity of enzyme, hydrolysis time, molecular weight and bioactivity, as well as AA composition. Additionally, pre-treatment was needed to enhance hydrolysis as the cleavage sites were exposed with restrictions to higher exposure to heat, resulting in AA structures were deformed. Table 4 shows resulting barriers in the industrial sector due to it cost-ineffectiveness [25].

Table 3. Protein content (%), extraction yield (g/100ml), and degree of hydrolysis (%) of unhydrolysed (UH), enzyme hydrolysed (EH), and acid hydrolysed pigeon pea (AH) pigeon pea. *Different superscripts in the same column for one sample show the significant differences of $p \leq 0.05$.

Sample	Protein content (%)	Extraction yield	Degree of hydrolysis (%)
UH	20.07 ± 0.8^{a}	2.38 g/100mL	34.32 ± 0.57^{a}
AH	24.05 ± 0.5^{b}	1.64 g/100mL	62.77 ± 0.66^{b}
EH	22.05 ± 0.5^{b}	2.50 g/100mL	68.42 ± 0.48^{c}

Table 4. Comparison of operating costs of different processing types of pigeon pea protein hydrolysates. The two methods compared were acid hydrolysis (AH) with enzymatic hydrolysis (EH).

Operating cost	Acid Hydrolysis	Enzyme Hydrolysis
Pigeon Pea protein hydrolysate production		
Ethanol (USD/L)	0.79	0.79
Roasting (kWh)	0.045	0.045
Grinding (kWh)	0.045	0.045
Total reaction time (h)	1.5-2	3
Hydrochloric Acid (USD/L)	2.77	-
Papain (USD/kg)	-	52.6
Protein yield (kg/L)	16.4	25
Total operating cost (USD/kg)	3.65	52.48

The study economic viability was assessed using a range of economic measures, one of the most important of which was the cost of manufacturing, sample preparation and total running expenses. The total operating costs was 3.65 USD/kg for AH and 52.48 USD/kg for EH, respectively (Table 4). With its competitive overall operative costs and good protein recovery yields, AH clearly was as the most economically viable agent.

3.4. Anti-nutritional factors

Tannic acid equivalents (mg·g^{-1}) were assessed as 7.07, 6.46, 5.41 and 4.8 in unhydrolyzed, defatted and hydrolysed pigeon peas, respectively (Figure 1A). A study reported the range of tannin in unprocessed pigeon pea as 4.3–11.4 mg·g^{-1} [26]. Tannin-protein complexes have been suggested to cause decreased AA availability, increased fecal nitrogen, inadequate protein digestibility and decreased iron bioavailability. These complexes might not be separated, which caused them as eliminated with the wastes [27]. Hydrolysates effectively eliminated tannins from the protein complex and improved nutritional values of the hydrolysates. The total phenolic content was assessed as 1.88 mg 100 g-1, 1.504 mg·100 g-1, 1.45 mg·100 g-1 and 1.376 mg·100 g-1 for unhydrolyzed, defatted, acid and enzymatic hydrolysates, respectively (Figure 1A). Prior investigations reported 1.6 mg·100 g-1, which verified the reported values [26]. After hydrolysis (acid or enzyme), the OH group decreased significantly and enhanced bioavailability of the macronutrients, as revealed in similar studies where heat and acid treatments decreased quantity of the free form as well as bound phenolic compounds that even leached out in water, ultimately making dormant sites of the proteins active [28]. Phytic acid levels were assessed as 7.13, 6.175, 5.415 and 4.7 mg·g-1 for unhydrolysed, defatted, acid and enzymatic hydrolyzed forms, respectively (Figure 1A). A study reported that the phytic acid content of pigeon pea was assessed from 4.87 to 6.54 mg·g-1, similar to the current findings [26]. Based on studies, total free (unbound) mineral contents increased after the whole wheat bread was autoclaved and microwaved simultaneously and the quantity of phytic acid decreased [29].

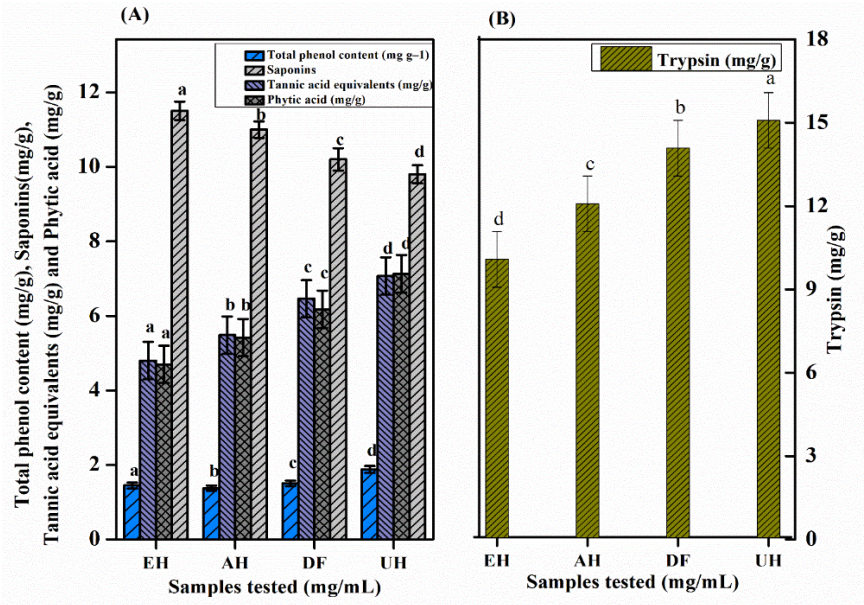

Figure 1. Anti-nutritional properties of unhydrolysed (UH), defatted (DF), and acid hydrolysed pigeon pea (AH). (A) Total phenolic content, saponins, tannic acid equivalents and phytic acid (B) Trypsin inhibition assay.
*Values are mean ± standard deviation (SD); bars with different letters have significantly different mean values of $p \leq 0.05$.

Saponins belong to a complex class of naturally occurring triterpenes or steroidal glycosides, detected in numerous types of plants, including oil seeds and pulses. Since saponins change permeability of the cell walls, they may result in adverse effects when consumed. The small intestine cells are bound by saponin, which alters how nutrients are absorbed through the intestinal membrane [1]. Saponins were assessed to be 9 mg.g^{-1} for unhydrolyzed and 11.5 mg.g^{-1} for the enzymatic hydrolysates. Similar values were reported in previous studies by Sekhon et al. [26]. Saponins include positive correlations with HRSA. This study highlighted that similarly hydrolysates included higher values than those unhydrolysates did. Saponins are well known for their capacity to yield foam that is exceptionally stable [3]. Trypsin inhibition activity of the unhydrolyzed samples was lower than that of hydrolysed samples (Figure 1B) [30]. After hydrolysis of H-bonds, trypsin-protein complexes were undigested, leading to decreases in inhibitors. These studies justifies relevance of the assessment of inhibitors as they delay the metabolism; hence, it must be addressed while consuming legumes as the source proteins. It is reported that trypsin-protein complexes are undigested after the lysis of H-bonds and decreases in inhibitors [21]. Studies on trypsin protease inhibitors (tannins, phytates, trypsin inhibitors and goitrogens) have shown their interference with the digestion processes and production of pancreatic hypertrophy or hyperplasia, inhibiting growth of the epithelial cells that lining mucosa and thus including anti-nutritive effects. Various methods have been used in food processes to address effects of these food anti-nutrients, including milling, soaking, germination, autoclave and microwave treatments and fermentation [31].

3.5. Fourier transform infrared spectrum analysis

Secondary structure of the proteins was assessed using FTIR spectroscopy [30]. Figure 2A shows that spectral characteristics of the globular complex (GC) proteins in unhydrolyzed samples were the Amide-A band at approximately 3274.31 cm^{-1} (NH stretching), the Amide-B band at approximately 2854.99 cm^{-1} (C-H symmetric stretching modes of methyl), the amide-I band at 1743 cm^{-1} (C=O carbonyl stretching), the amide-II band at 1634 cm^{-1} (C=C aromatic stretch) and N–H bending at 1540 cm^{-1} and the aromatic C-H in plane bend at 1074.88 cm-1. These findings were consistent with the previous studies [17, 31]. The AH spectra showed characteristic peaks in the range of 3273–2874.17 cm^{-1}, indicating that respectively Amide A (N-H and C-H stretching) and Amide B (methyl C-H stretching) bands brought on by the bending resonances of the intra and intermolecular hydrogen bonds (Figure 2B). Peaks at 3231.46, 3065.45, 2958.78 and 2875.78 cm^{-1} showed that Amides A and B were visible in the IR spectra of EH (Figure 2C). The aromatic C-H out of the peaks were visible at 666.10 and 1398.04 cm-1 indicating N-O aliphatic stretch. Moreover, peak of 1451.70 cm-1 showed visible peaks of C-H asymmetric bend, and 1579.13 cm-1 showed a band of the NH bend (Figure 2C). The connection between functional groups of C-O and N-H forms a helical structure [32].

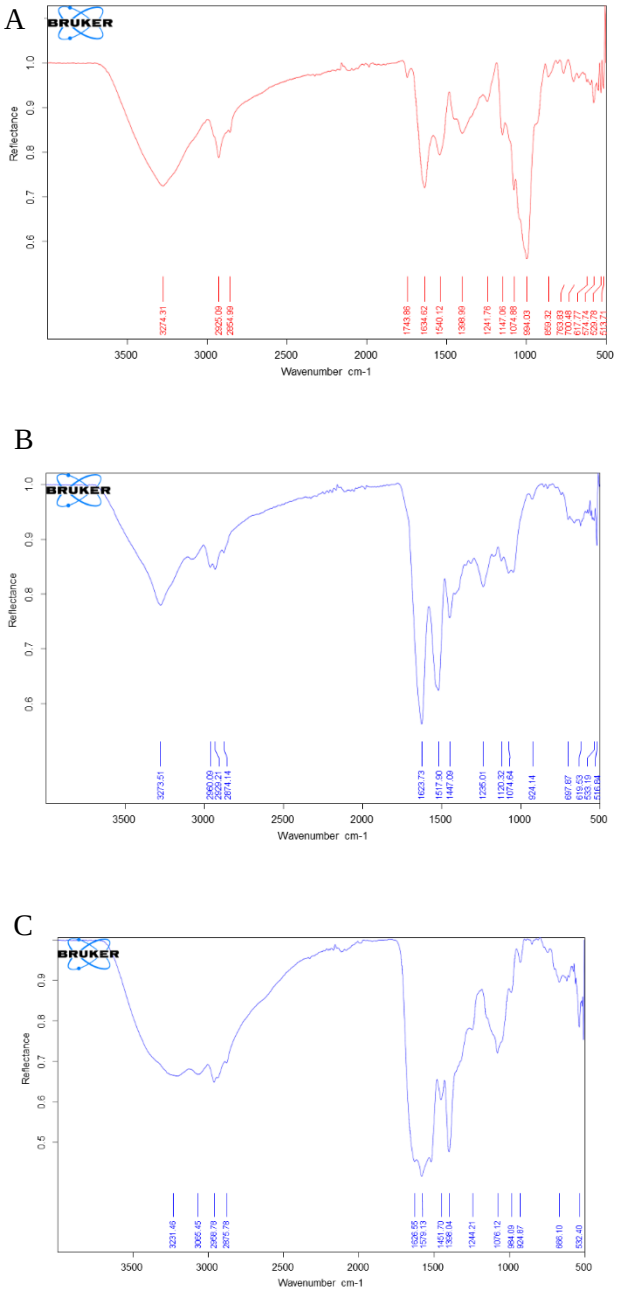

Figure 2. Fourier transform infrared (FTIR) spectra of (A) unhydrolysed (UH), (B) acid hydrolysate (AH) and (C) enzymatically hydrolysed (EH) of pigeon pea.

The conventional protein bands of Amide I (1600–1700 cm^{-1}), Amide II (1500–1580 cm^{-1}) and amide III (1200–1400 cm^{-1}) showed visible bands of Amides A and B. A previous study showed that the characteristic protein bands were linked to specific stretching and bending vibrations of

the protein backbones. The α-helix (1650–1658 cm^{-1}) and β-sheet (1638–1687 cm^{-1}) were the primary contributors to Amide I, while Amide II was mostly caused by N-H bending vibrations (60%) connected to C-N stretching vibrations (40%), representing dominance of the protein contents [33]. Therefore, peaks (1300, 1650 and 1688 cm^{-1}) investigated in the hydrolysate spectra were similar to α-helix and β-sheet [4, 17].

3.6. Protein Profile Analysis (SDS-PAGE)

In vertical gel electrophoresis, proteins are separated based on their molecular weights. In this study, acid and enzymatically hydrolyzed pigeon pea samples were subjected to SDS-PAGE (15%) to assess molecular weights of the hydrolysates. Protein marker (Lane 1) ranged 245–11 kDa which included various molecular weights, followed by acid samples in Lane 2, unhydrolysed samples in Lanes 3 and 4 and enzymatic hydrolysates in Lane 4. Acid hydrolysed samples showed distinct bands in the range of 250–20 kDa (Lane 2), which were suggested as albumins and globulins and the difference in molecular weights showed the extraction procedure, making them high-molecular weight (HMW) peptides. The control unhydrolyzed protein demonstrated intense bands at 135–100 kDa (Lanes 3 and 4). The SDS-PAGE analysis of the EH revealed generation of low-molecular weight (LMW) peptides with a mass range of less than 15 kDa (Figure 3, Lane 5).

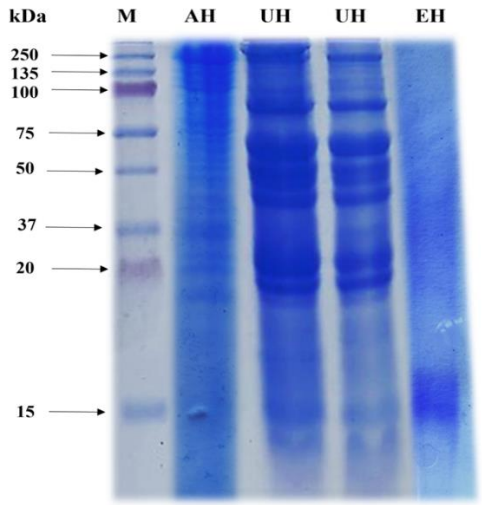

Figure 3. SDS-PAGE electrophoresis of samples before and after hydrolysis. Lane 1-molecular weight standards (250-15kDa), Lane 2- acid hydrolysed (AH), Lane 3 and 4- unhydrolysed (UH), Lane 4-enzymatically hydrolysed (EH).

Figure 3 illustrates differences in bands affected by hydrolysis differently. Thus, it could be assessed that LMWs were produced by breaking the complex protein structure and peptide bonds by use of AH conditions while enzymatically hydrolysed samples included the presence of LMW peptides, indicating complete hydrolysis of the pigeon peas. A similar LMW band was seen in casein and flaxseed hydrolysate studies [10, 11].

3.7. Amino acid composition

The AA compositions of pigeon pea unhydrolysed (UH), acid (AH), and enzymatic hydrolysates (EH) are shown in Table 5.

Table 5. Amino acid profile of pigeon pea, UH (unhydrolysed), AH (acid hydrolyzed), and enzymatic hydrolysed (EH) pigeon peas.

	Composition of amino acid (g/100g)		
	Essential amino acids		
Amino acid	UH	AH	EH
Histidine	4.2	4.45	4.65
Isoleucine	3.25	3.42	3.62
Leucine	7.54	8.21	8.91
Lysine	7.25	7.35	7.65
Methionine	1.54	0.85	0.74
Phenylanine	7.21	8.75	8.95
Threonine	3.98	3.1	3.87
Tryptophan	0.56	0.1	0.2
Valine	4.15	3.25	3.85
	Non-essential amino acids		
Alanine	4.2	3.95	4.05
Arginine	6.98	6.85	7.1
Aspartic acid	10.56	11.2	10.21
Cystine	0.98	0.38	0.55
Glutamic acid	18.24	20.02	19.56
Glycine	3.25	3.1	3.32
Proline	5.2	4.2	4.56
Serine	4.25	6.04	6.2
Tyrosine	2.12	2.45	2.54
EAA	39.68	39.68	42.26
NEAA	55.78	50.09	58.09
AAA	9.89	11.3	11.69
HAA	37.88	36.21	38.4
SAA	2.52	1.23	1.29
PCAA	18.43	18.65	19.4
NCAA	28.8	31.22	29.77

EAA, essential amino acids; NEAA, non-essential amino acids; AAA, aromatic amino acids; HAA, hydrophobic amino acids; SAA, sulphur containing amino acids; PCAA, positively charged amino acids; NCAA, negatively charged amino acids

The current composition supports protease ability to specifically cleave proteins at peptide bonds that hydrophobic AAs contribute to, increasing quantity of the linked AAs [16]. The pigeon pea unhydrolysed AA profile showed that the legume was rich in proteins and included significant quantities of EAAs that were better than the needs of the World Health Organization/Food and Agriculture Organization/United Nations University (FAO/WHO/UNU) Expert Consultation [10]. Findings from this study revealed greater concentrations of hydrophobic AAs (38.4 and 37.88%), EAAs (39.68, 42.26%), sulphur containing AAs (2.52 and 1.29%) and aromatic AAs (9.89 and 11.69%) for unhydrolyzed and enzymatic hydrolyzed samples, respectively. These were

similar to previous findings by Nwachukwu et al. for the flaxseed protein and thermoase hydrolysates (8.62 and 9.03% of aromatics; 34.55 and 35.72% of hydrophobics) [34].

Comparison of AA profiles of the protein hydrolysates derived by the isoelectric precipitation method to native protein (unhydrolysed pigeon pea) revealed a little detectable variation, suggesting that the protein composition was largely unaffected by the extraction process [30]. Olagunju et al. [14] detected that use of distinct proteases during the hydrolysis of pigeon pea protein produced similar findings. The hydrolysates included higher concentrations of particular AAs, compared to those the non-hydrolyzed protein did, including glutamic acid, histidine, leucine, isoleucine, phenylalanine, arginine, tyrosine, and tryptophan. Glutamic and aspartic acids were the most frequent AAs in AH and EH, whereas levels of tryptophan and methionine were below the recommended levels. Branching-chain amino acids (BCAAs) and a majority of hydrophobic AAs (glycine, alanine, valine, leucine, isoleucine, proline, and phenylalanine) increased upon enzymatic degradation using protease enzymes.

3.8. Functional characteristics of bioactive peptides

3.8.1. Water and oil absorption capacities

Technically, WAC and OAC are pH dependent and detrimental parts of this study. The WAC values of the hydrolysates were higher than those of unhydrolysed samples. At pH 4, sharp decreases were seen in WAC and OAC values due to the pH near the isoelectric point of the proteins (Figure 4A). Similar findings were reported by the literatures, showing that peptides typically included higher water-holding capacities than those of their associated flour forms. This finding was similar to another finding and likely resulted from changes in protein conformation that exposed further water-binding sites, increasing protein polarity, electric charge and proportion of the proteins bound in water [4, 17]. The OAC value is important as an estimate of oil ability to absorb proteins, which can indicate how hydrophobic a protein is. The non-protein components in flours, including starch granules and lipids, may partially function as a barrier to water penetration, fractions with higher protein contents with fibers include greater potentials to hold water. Due to their lower lipid contents and smaller particle sizes, lentil protein peptides included better water-holding capacities than those the lentil flours did.

The OAC of pigeon pea unhydrolysed seeds was lower than that of the enzymatic hydrolysate with significant general decreases at pH 4 (Figure 4A). Compared with other spectra of legumes, these values were at average levels, as reported in a study of protein hydrolysates with higher surface hydrophobicity and better surfactant characteristics. However, denaturation of the globular proteins might expose hydrophobic regions. It is believed that OAC is created by the binding of non-polar side groups of proteins, leading to oil entrapment. The OAC is therefore affected by the quantity of hydrophobic AAs that are exposed, as well as the quantity of hydrophobic AAs in the proteins that can visibly be demonstrated in the AA profile (Table 5) for the unhydrolysed and hydrolysed samples [3, 20, 35]. Based on a study, globular proteins with further hydrophilic and polar AA residues on the surface included a higher WAC and proteins with further hydrophobic and non-polar AA residues might include a higher OAC [36]. Plant-based proteins with good water and oil absorption characteristics can create plant-based protein analogues and emulsions, which are beneficial in food industries.

3.8.2. Emulsifying capacity

The EAI (Figure 4B) and ESI (Figure 4C) values of the hydrolysed samples were higher than those of unhydrolyzed and defatted samples, which was observed with pH as a critical factor. The lowest EAI and ESI values were reported at pH 4, which was near the isoelectric point of soy and whey proteins and included a lesser solubility based on Figures 4B and 4C, respectively. This indicated that pH substantially affected emulsifying characteristics of the peptides with and without hydrolysis. Stability of the emulsion increased by pH levels that were below the isoelectric point. The maximum EAI and ESI values for the emulsions at pH 8 are shown in Figures 4B and 4C [19]. It is shown that the hydrophilic/hydrophobic AA composition and equilibrium development of the interfacial film, as well as the solubility and flexibility of the protein molecules, are interrelated to differences in EAI and ES for various proteins [37]. Protein isolates with higher EAI and ES additionally included further hydrophilic AA residues and were further soluble (Figures 4B and 4C). Amphiphilicity of the peptides includes major effects on the emulsifying abilities of hydrolysates. Based on multiple studies, hydrolyzed peptides enhance hydrolysates abilities to emulsify due to their potentials to unfold at the oil/water interface [37]. Additionally, they are more likely to include residues that interact with the aqueous phase and the oil droplets, respectively, whether they are hydrophobic or hydrophilic [2, 14]. Due to steric effects, this interaction makes the emulsion further stable [38]. The AH offers further potentials for food emulsion uses, including yoghurt, mayonnaise and ice cream since EAI and ESI are essential indices for food emulsion use and quality control. Moreover, higher molecular weight of the protein hydrolysates may result in higher emulsion capacities of AH.

3.8.3. Foaming characteristics

Proteins in dispersions decreased surface tension at the water-air interface, resulting in the ability to foam. The hydrolysates half-life could be changed by the preservation of the viscoelastic adsorbed layer, suggesting that FS might necessitate addition of functionality improvers to the hydrolysate. It is well known that globular proteins frequently produce adsorbed layers that are elastic with enhanced viscosity and hence support stability of the foam [39]. Foaming capacity and stability are pH dependent. The FC decreased at pH 4 and reached its maximum value at pH 6 (decreased in alkaline conditions). The FC was inversely proportional to pH. At pH 6, net charge increased, which led to increases in foaming capacities. At pH 4, FC achieved a lower solubility at its isoelectric point. Increased concentrations of amphiphilic peptides contributed to increased FC (Figure 4D). Moreover, FS depended on protein-protein interactions with the matrix [20]. An extensive intermolecular network (protein-protein interactions) preserved by proteins maintained their tertiary structures at the interface, resulting in strong films and further stable foams.

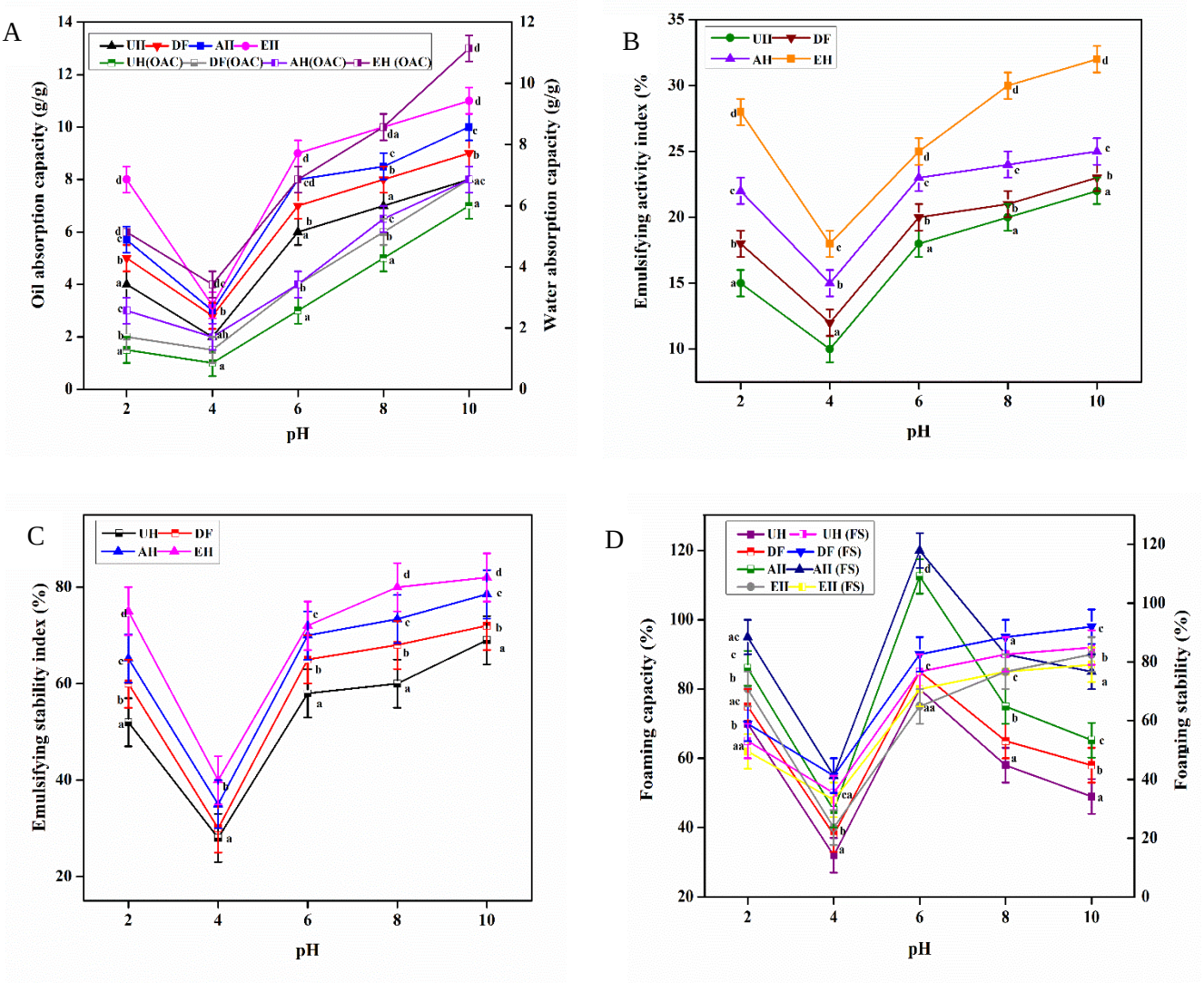

Figure 4. Functional properties of unhydrolysed (UH), defatted (DF), acid hydrolysed (AH) and enzymatic hydrolysed (EH) of pigeon pea. (A) WAC (Water absorption capacity), OAC (Oil absorption capacity); (B) EAI (Emulsifying activity index); (C) ES (Emulsion stability); (D) FC and FS (Foam capacity and stability) (i.e., foam volume vs. time respectively.

*Values are mean ± standard deviation (SD); error bars with different letters have significantly different mean values of $p \leq 0.05$.

Protein LMWs and other structural characteristics helped generate foams further quickly. However, they might not be effective for creating protein-protein interactions that resulted in stable foams (Figure 4D). These reports help explain why the unhydrolysed control pigeon pea included a small foam ability (expansion of foam) [37]. Large concentrations of hydrophobic AAs could be the reason for decreased FC values. Stability of the foam formation was affected by the strength of protein films and permeability for gases [18]. Several peptides with various hydrophobicity, charge balances and conformations from the native molecule are created during the digestion of proteins. These peptides are more flexible due to their lower molecular weight; hence, a stable interfacial layer is seen and the rate of diffusion to the interface increases, improving the foamability characteristics. For a protein to foam effectively (e.g. including high foamability), it should adsorb quickly during the transient stage of foam formation. These findings revealed increases in surface activity, most likely due to the originally larger surfaces created by partial proteolysis that allowed for the incorporation of further air [39].

3.9. Antioxidant activities of peptides

Several chemical or biological assays are needed to assess antioxidant mechanism and quantify antioxidant activity of the legume extracts [40]. Although this in-vitro study is fascinating from a strictly predictive viewpoint based on chemical processes, it might sometimes correspond to in-vivo systems. The MCA and HRSA are based on electron transfer reactions (ET) and hydrogen atom transfer reactions (HAT), respectively; DPPH is a hybrid HAT and ET-based assay [7].

3.9.1. The DPPH radical scavenging activity

Due to the method simplicity, efficacy and credibility, DRSA is often used to assess antioxidant capacity of foods, peptides and biological samples. In the present study, DRSA in pigeon pea samples was 38.60, 42.45, 74.25 and 80% for unhydrolysed, defatted, acid and enzymatic hydrolysates, respectively (Figure 5A). Results were almost similar to those of literature. The DRSA of hydrolysates was able to achieve a similar efficiency to that of GSH, which is the predominant intracellular antioxidant that helps in keeping redox equilibrium in the body and as well as modulating cell viability. Other studies have reported similar increase in antioxidant activity with increase in DH. Increased hydrolysis led to further hydrophobic side chains exposed, improving DRSA. Moreover, positive correlations were recorded between the phenolic content and DRSA, which justified the oxidative stress decreases on cellular levels. Relatively, as phenolic substances or their respective levels of hydroxylation increase, their abilities to scavenge DPPH radicals increase as well. Free radicals combine with cellular components such as DNAs, proteins and cell membranes; hence, these metabolites (primary and secondary) solve the problem of oxidative stress by neutralizing and disguising them as harmless agents through biological processes [10, 40].

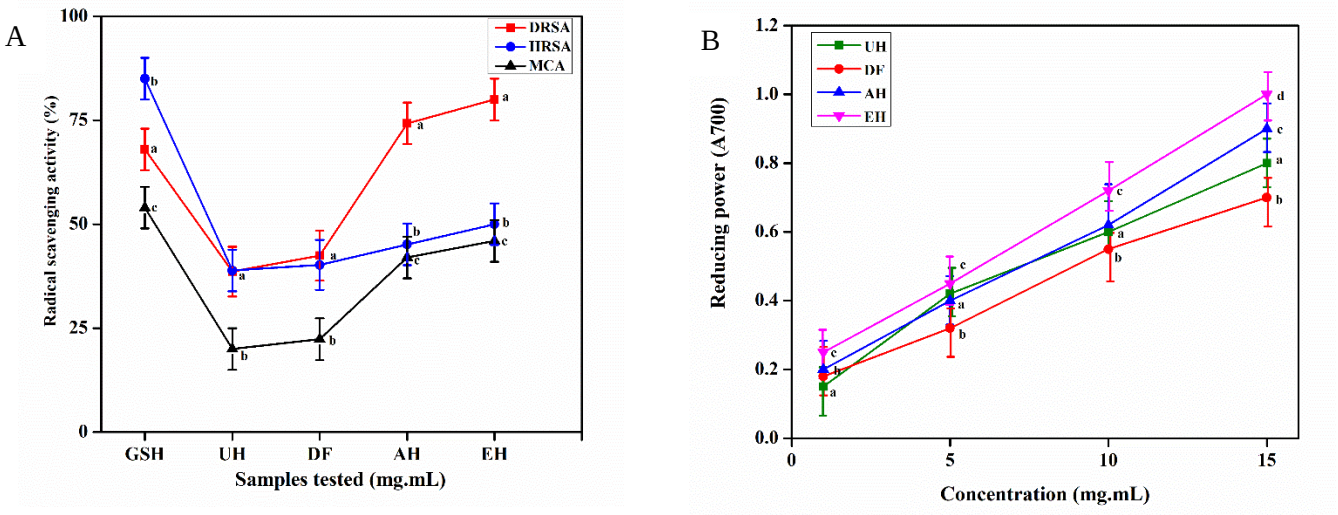

Figure 5. (A) Antioxidant inhibition activities (DPPH, HRSA and MCA) of unhydrolysed (UH), defatted (DF), acid hydrolysed (AH) and enzymatically hydrolysed pigeon pea (EH) and (B) Reducing power of unhydrolysed (UH), defatted (DF), acid hydrolysed (AH) and enzymatically hydrolysed pigeon pea.
*Values are mean ± standard deviation (SD); bars have significantly different mean values of $p \leq 0.05$.

3.9.2. Hydroxyl radical scavenging activity

Technically, HRSA assesses scavenging ability of peptides when hydroxyl radicals are generated due to the reaction between H_2O_2 and metal ions such as Fe^{2+} ion form. Hydroxyl ion includes short lifespan and highly reactive species that degrades macromolecule proteins, DNAs and lipids. Hydroxyl ion is addressed as a responsible factor for initiating lipid oxidation processes, causing cell damages that result in ageing and other chronic illnesses. Scavenging abilities of pigeon pea unhydrolyzed, defatted, acid, and enzymatic hydrolyzed samples were 38.55, 40.2, 45.15, and 50%, respectively (Figure 5A). In the current study, HRSA showed similar results to those of DPPH and phenolic contents did in the samples. Total length of the peptides, makeup and sequencing of the AAs and other variables alter antioxidant activity of protein hydrolysates. It can be concluded from the results that hydrolysis enhanced the hydroxyl radical scavenging capacity of pigeon pea proteins; similar to that observed in the enzymatic hydrolysis of pigeon pea using various enzymes [23].

3.9.3. Metal ion-chelating assay

Transition metal Fe^{2+} stimulates production of OH- and superoxide radicals (O_2), triggering oxidative chain reactions. Chelating chemicals regulate the oxidative chain reactions caused by the radicals and decrease quantity of transition metals available in organic materials. The Fe^{2+} Scavenging ability of the hydrolysed samples was similar to that of GSH and significantly better than that of unhydrolysed samples [8]. The current study indicated that metal chelating abilities were high; similar to other studies that verified hydrolysate antioxidant abilities could inhibit oxidant reactions and help in prolonged storage. Increased hydrolysed peptides or polar AA concentrations might be associated with increased Fe^{2+} chelating activity within hydrolysis. Protein hydrolysate ability to chelate metal ions is generally reported responsible for their abilities to bind with ions. It has been stated that histidine-containing peptides include metal-chelating activities via their imidazole rings. Another significant reason for antioxidant activity included ability to bind transition metals because transition metal ions such as Fe^{2+} and Cu^{2+} naturally stimulate creation of reactive oxygen species (ROS) such as hydroxyl radical (OH), triggering oxidation of fatty acids [23].

3.9.4. Reducing power

The reducing power of a compound is studied to assess its antioxidant potentials. Figure 5B demonstrates the reducing power reached by acid-base hydrolysis for 5 h at various doses (1–15 mg·mL^{-1}). Hydrolysates included the highest reducing power, showing that these hydrolysates in EH followed by AH included further bioactive peptides that could scavenge free radicals. The activity assessed by UH was the lowest. Reducing power of the samples increased with a higher absorbance value. When reducing substances were present, the Fe^{3+}/ferricyanide complex was reduced to the ferrous form (Fe^{2+}) through electron donation. Briefly, quantity of Fe^{2+} was measured at 700 nm [7]. Variation in reducing power might be a significant characteristic, indicating changes in AA composition after hydrolysis. Information suggested that hydrolysates might help free radicals by donating one electron [8].

3.9.5. Anti-diabetic assay

In general, α-amylase enzyme in hydrolysis of glycosides is responsible for the breakdown of complex carbohydrates into oligosaccharides and glucose for a better uptake. Hence, inhibition of such an enzyme is helpful in decreasing breakdown of complexes to simpler levels, making it a better strategy to manage diabetes. For this reason, naturally occurring bioactive substances in foods that are helpful in treatment of diabetes have widely been reported. Figure 6 shows that various samples included anti-diabetic activities. The synthetic drug glyciphage showed the highest inhibition rate (56%), whereas hydrolysates EH (44%) and AH (38%) showed the best inhibitory effects, compared with the activity of UH (18%) and DF (22%). Low activities of the UH and DF might be due to weaker bond interactions, including electronic and hydrophobic bonds with lesser bond availability to bind with

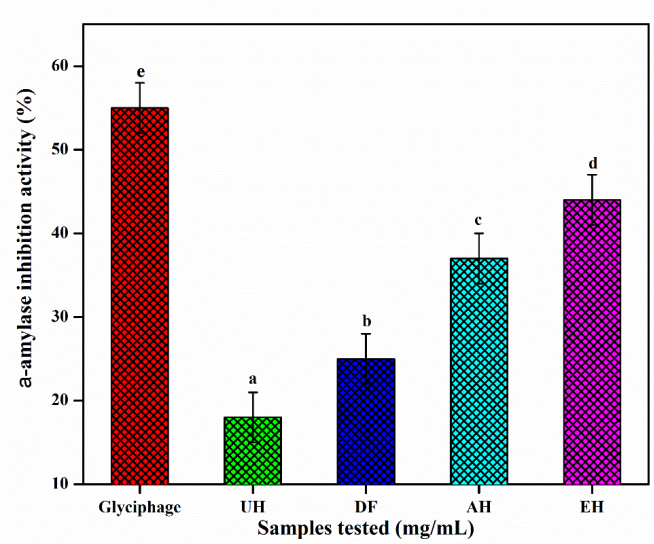

Figure 6. Inhibition of α-amylase activity of unhydrolysed (UH), defatted (DF), acid hydrolysed (AH), and enzymatically hydrolysed pigeon pea (EH) at absorbance 540 nm.

*Values are mean ± standard deviation (SD); bars have significantly different mean values of $p \leq 0.05$.

polysaccharides and dietary fibers, ultimately leading to lesser inhibition. After hydrolysis, synergistic activities between the peptides and inhibition were recorded. Results

were similar to those of various hydrolysates. Although studies have shown certain differences in inhibition due to the sources of raw materials, filtration process and type of hydrolysis, inhibiting activity of α-amylase may lessen the likelihood of hyperglycemia in the body [14].

4. Conclusion

This study highlights synthesis of functional proteins with comparative findings of the various methods. Effectiveness of pigeon pea as a potential alternative protein source was the major finding of this study as well as assessment of the peptide functional, antioxidant and anti-diabetic characteristics. This study has demonstrated use of acid and enzymatic hydrolysis methods for the extraction of pigeon pea protein hydrolysates to subdue anti-nutritional factors that delay efficacy of metabolism. This approach can be used as an excellent alternative to traditional proteins with a higher concentration of bioactive peptides. It has been observed that the bioactive peptides include beneficially functional qualities such as helping in developments of emulsions, gels and suspensions, making them ideal choices for potentially commercial protein powders. This study provides numerous novel possibilities for research on plant-based protein sources and pigeon pea can be used as a vegan source while preserving the protein texture, flavor and nutritional profile. These help in treating various chronic diseases and metabolic disorders; hence, contributing significantly to health and food industries. The present study addresses usethe use of cheaper vegan protein sources in human diets.

5. Acknowledgements

None.

6. Conflict of Interest

The authors declare no potential conflict/competing interest.

7. Authors Contributions

Conceptualization, N.S.; methodology, J.M.; software, J.M.; validation, N.S..; formal analysis, J.M.; investigation, J.M.; resources, N.S.; data curation, writing—original draft preparation, J.M..; writing— review and editing, N.S. and V.G.P.; visualization, N.S.; supervision, N.S.

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ارزیابی ویژگی‌های فیزیکوشیمیایی، ضداکسایشی، ضد دیابتی و تغذیه‌ای ترکیبات حاصل از آبکافت پروتئین لوبیا کفتری (سودانی) در شرایط برون تنی

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چکیده

سابقه و هدف: دانه‌های *Cajanus cajan* (لوبیا کفتری یا سودانی) به لحاظ ویژگی‌های خاصی که دارند می‌توانند به‌عنوان منابع جایگزین پروتئین گیاهی مورد استفاده قرار گیرند. هدف از این مطالعه بررسی پپتیدهای فعال زیستی در نخود کفتر با استفاده از روش مقرون به صرفه آبکافت[1] اسیدی و آنزیمی بود.

مواد و روش ها: در این مطالعه، لوبیا کفتری به دو روش اسیدی و آنزیمی آبکافت شد. میزان و راندمان پروتئین، درجه آبکافت، پروفایل ترکیبات ضدتغذیه‌ای، طیف بینی مادون قرمز تبدیل فوریه[2]، تجزیه و تحلیل ترکیبات حاصل از آبکافت با بررسی محتوای پروتئین و بازده، درجه هیدرولیز، مشخصات ضد تغذیه‌ای، طیف سنجی مادون قرمز تبدیل فوریه، سنجش آنتی اکسیدانی ۲،۲-دی فنیل-۱-پیکریل هیدرازیل[3]، آزمون مهار رادیکال هیدروکسیل[4]، آزمون کیلیت یون فلزی[5] و قدرت احیاکنندگی[6] انجام شد. علاوه بر این، اثرات ضد دیابتی با استفاده از روش مهار آلفا آمیلاز[7] مورد مطالعه قرار گرفت.

یافته‌ها و نتیجه‌گیری: آبکافت لوبیا کفتری با هضم اسیدی (pH 4) و آنزیم انجام شد و سپس به‌منظور دستیابی به بخش پپتیدی زیست فعال از فیلتراسیون غشایی استفاده شد. لوبیا کفتری آبکافت شده در مقایسه با شاهد، محتوای پروتئین و درجه آبکافت خوب و بیشتری داشت. درجه آبکافت برای روش اسیدی ٪ ۶۲/۷، برای روش آنزیمی ٪ ۶۸/۴۲ و برای بخش آبکافت نشده ٪۳۴/۳۲ بود. نمونه های آبکافت شده در آزمون طیف بینی مادون قرمز تبدیل فوریه پیک‌هایی در محدوده cm^{-1} ۳۵۰۰-۴۰۰۰ داشتند که نشان دهنده وجود آمیدهای I و II می‌باشد. وزن مولکولی پپتیدهای حاصل از آبکافت اسیدی محدوده وسیع‌تری (۲۵۰-۲۰ کیلو دالتون) در مقایسه با بخش‌های آبکافت آنزیمی (EH) با وزن مولکولی بسیار پایین کمتر از ۱۵ کیلو دالتون داشتند. پپتیدهای حاصل از آبکافت اسیدی (AH) ویژگی‌های زیست فعال قابل توجهی در مقایسه با ویژگی‌های آنتی اکسیدانی و ضد دیابتی پپتیدهای آبکافت آنزیمی در برابر استانداردها نشان دادند. این مطالعه تولید ترکیبات حاصل ازآبکافت پروتئین لوبیا کفتری با دو روش سنتی (اسیدی) و جدید (آنزیمی) برجسته می‌کند و نشان می‌دهد که آبکافت اسیدی می‌تواند روشی ارزان و اقتصادی برای تولید ترکیبات حاصل از آبکافت پروتئین با ویژگی‌های زیست فعال خوب باشد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[1] Hydrolysis
[2] Fourier transform infrared spectroscopy
[3] 2,2-diphenyl-1-picryl hydrazyl (DPPH)
[4] Hydroxyl radical scavenging assay
[5] Metal chelating ion assay
[6] Reducing power
[7] α-amylase inhibition assay