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Antioxidant polypeptides derived from pigeon pea (*Cajanus cajan* (L) Mill sp.) by enzymatic hydrolysis

[1,*]Siriporn, B., [1]Thongkorn, P., [1]Waraporn, S., [2]Wiriyaporn, S., [1]Sinee, S., [2]Chiramet, A. and [3]Rotimi, E.A.

[1]Researcher of Expert Center of Innovative Health Food, Thailand Institute of Scientific and Technological Research, Pathum Thani, Thailand, 12120

[2] Researcher of Expert Center of Innovative Herbal Products, Thailand Institute of Scientific and Technological Research, Pathum Thani, Thailand, 12120

[3]Department of Food and Human Nutritional Sciences, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada

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Abstract

Pigeon pea (*Cajanus cajan* (L) Mill sp.) seeds are rich sources of protein in the legume family and their consumption has been associated with the prevention of non-communicated diseases, which is attributable to their content of bioactive components. Antioxidant protein hydrolysates were produced from pigeon pea protein isolate (PPI) by enzymatic hydrolysis using pancreatin and flavourzyme. The hydrolysates were analyzed for their physicochemical, molecular weight, amino acid composition, and *in vitro* antioxidant activities. The molecular weights of polypeptides in the hydrolysates were 8, 20, 25 and 48 kDa, which were determined after pancreatin or flavourzyme hydrolysis of the protein isolate for 4 h. Pancreatin-hydrolyzed pigeon pea protein (PPHP) contained high hydrophobic amino acids, especially isoleucine, leucine and valine, which were related to the high content of aromatic amino acids. The hydrolysates obtained from flavourzyme hydrolysis of pigeon pea proteins (PPHF) presented significantly higher capacities to scavenge $ABTS^{\cdot+}$ and reduce Fe^{3+} better than that of PPI, while the PPI exhibited strong DPPH scavenging (98.4 mg trolox equivalent antioxidant capacity). The results indicated that the partial hydrolysis for PPI provided medium to high molecular weight of peptides. Therefore, PPHF could be a promising source of bioactive peptides and a potential ingredient for the formulation of functional foods against oxidative stress.

1. Introduction

Pigeon pea (*Cajanus cajan*) is a dry leguminous crop with fast-growing (6-9 months) season and adaptable to cultivation in tropical and subtropical climates (Adenekan *et al*., 2017; Abebe, 2022). Pigeon pea seeds commonly contain about 17.9-4.3% protein (170.37 to 251.16 mg/g of total soluble protein) and measurable amounts of essential amino acids and minerals based on cultivar type (Adenekan *et al*., 2017; Abebe, 2022). Protein hydrolysates and peptides especially bioactive peptides are a group of organic compounds formed of 2 to 100 amino acid units, which have many health-promoting properties such as immunomodulatory, antihypertensive, antimicrobial, insulin-mimetic, or antioxidant effects (Sbroggio *et al*., 2016; Czelej *et al*., 2022). Particle size, amino acid composition and sequence, and hydrophobicity are factors attributed to the structure of peptides while for antioxidant activity, peptides with smaller mass (≤3 kDa) have stronger potency than longer-chain peptides (Czelej *et al*., 2022). Moreover, the protein property would be limited by the tertiary structure due to the many antioxidants and amino acids that could be buried within the protein core (Sbroggio *et al*., 2016).

Enzymatic hydrolysis is greatly favoured over the chemical method because it requires mild reaction conditions, generating few undesirable side products, and facilitating high yield and product quality (Czelej *et al*., 2022). Enzymatic hydrolysis is considered a particularly important method of breaking protein molecules into small peptides of various sizes, and eventually amino acids to generate bioactive peptides and could increase the antioxidant activity of peptides (Sbroggio *et al*., 2016; Czelej *et al*., 2022). Various proteinases such as alcalase, neutrase, pepsin, trypsin, seabzyme L,

*Corresponding author.
Email: *siriporn_c@tistr.or.th*

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viscozyme L, pancreatin, thermolysin, flavourzyme, protamex, or bromelain had been extensively reported to produce peptides with antioxidative properties (Czelej *et al*., 2022). However, the antioxidant properties of protein hydrolysates depend on several factors such as the type of protein substrate and enzyme, degree of hydrolysis, pretreatment of the substrate, the ratio of enzyme to substrate, the initial concentration of the proteins, processing time, pH, and temperature (Karamać *et al*., 2016; Sbroggio *et al*., 2016; Czelej *et al*., 2022). Hence, the objective of this research was to study the impact of the enzymes pancreatin and flavourzyme on the degree of hydrolysis, molecular weight, total amino acid, and antioxidant activity such as DPPH, ABTS, and FRAB of pigeon pea hydrolysates.

2. Materials and methods

2.1 Materials

Dehulled pigeon pea was purchased from a Food Security 4.0 store, Bueng Kum, Bangkok province. Protease from *Aspergillus oryzae* [Flavourzyme (≥500 U/g)] and pancreatin from porcine pancreas (4 × USP specifications) were purchased from Sigma-Aldrich, St Louis, MO, U.S.A. All other reagents were of analytical grade and purchased from Ajax FineChem part of Thermo Fisher Scientific (U&V holding Thailand, Nonthaburi, Thailand).

2.2 Extraction of dehulled pigeon pea protein

The preparation of dehulled pigeon pea protein was accomplished using the method described by Budseekoad *et al*. (2018) with modification. The peeled pigeon pea of 450 g was milled with 4500 mL distilled water using a high-speed grinder and then sieved through 250-micron filter. Alkaline extraction at pH 9 with 1 mol/L NaOH was applied, then stirred overhead stirred at 650-700 rpm for 2 hrs at room temperature. The supernatant was collected after centrifugation and then adjusted to pH 4.5 using 1 mol/L HCl followed by another round of centrifugation at 7,000 rpm, 4°C for 30 mins. The sediment was dispersed in distilled water at the ratio of 1:3 (w/v), adjusted to pH 7.0 with 1 mol/L NaOH, and then freeze-dried (Christ, Deta2-24LSC, UK). The freeze-dried sample was analyzed for physiochemical composition.

2.3 Enzymatic hydrolysis of dehulled pigeon pea protein

Two proteases (pancreatin and flavourzyme) were used individually for hydrolysis. Enzymes were added to freeze-dried extracts containing 50 g/L protein to obtain a final concentration of 2 g or mL of enzyme per 100 g protein. The hydrolysis condition was carried out using the optimum condition for each enzyme for maximum cleavage of pigeon pea protein isolate (PPI). Hydrolysis using flavourzyme was carried out at 50°C and pH 8.0 while pancreatin was carried out at 37°C, pH 7.0. All samples were hydrolyzed in individual reactor for 4 hrs (WP Winpact Fermenter FS-05, USA) at 300 rpm. All hydrolysis mixtures were placed in an autoclave at 95°C for 15 min (Zealway, GR 110DA, USA) to stop enzyme activity. Samples were allowed to cool at room temperature, filtrated with double layer cheese clothes, and the supernatant then freeze-dried (Christ, Deta2-24LSC, UK). The pigeon pea protein hydrolysates (PPHs) were further analyzed (Budseekoad *et al*., 2018).

2.4 Degree of hydrolysis

The degree of hydrolysis (DH) of pigeon pea protein hydrolysates (PPHs) was analyzed using the OPA (o-phthaldialdehyde) method with slight modification of the method described by Nielsen *et al*. (2001). The OPA is carried out by determining the α-amino nitrogen of peptides to estimate the percentage of cleaved peptide bonds in the protein sample. The total number of α-amino nitrogen in the sample was determined by complete acid hydrolysis using 10 mL of 6 M HCl for 1 g of sample at 110°C for 24 hrs. The absorbance of this OPA reagent and hydrolysates mixture was measured at 340 nm after incubation for 20 mins. The percentage DH was determined using the following equation:

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

Where h is the number of hydrolyzed bonds calculated (Nielsen *et al*., 2001) and h_{tot} is the value of constants, alpha and beta (soybean is referred). The constants, alpha and beta are different when different raw materials protein source (Adler-Nissen, 1986).

2.5 Physiochemical analysis

The yield of PPI, pancreatin-hydrolyzed pigeon pea protein (PPHP) and flavourzyme-hydrolyzed pigeon pea protein (PPHF) were calculated based on the ratio of the final weight of the dried protein extract to the initial weight of the dried protein. The protein yield recovery (%) was calculated by using the following equation (Wang *et al*., 1999).

% protein yield recovery = [weight (g) of PPI × protein content (%) of PPI]× [(weight of dry basis)×protein content (%) of dry basis]× 100

The samples were also analyzed for color in CIE Lab scale (L*, a* and b*) (Konica Minolta CR400, Japan), water activity (aw), moisture content (AOAC, 2000) and protein content (Dumas Combusion, Dumatec 8000 LECO, FP-528, USA).

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2.6 Amino acid profile of pigeon pea protein hydrolysate

The PPI, PPHP and PPHF were analyzed for amino acid composition using high-performance liquid chromatography (HPLC) amino acid analyzer (Shimadzu, model LC-20A). The samples (0.5 g) were digested using 6 M HCl at 110°C for 22-24 hrs. Digested samples were diluted with 10 ml of 0.1 mol/L HCl and the solution was then filtrated with membrane disc filter polytetrafluoroethylene (PTFE), for analysis. The amino acid was derivatized using post-column derivatizer (Shim-pack ISC-07/S 1504 Na) with three reagents, reaction solutions and o-phthaldialdehyde (OPA). The mobile phases consisted of three eluents: eluent A filled with 0.2 mol/L sodium citrate (containing 7% EtOH), pH 3.2; eluent B filled with 0.6 mol/L sodium citrate in 0.2 mol/L boric acid, pH 10; and eluent C filled with 0.2 mol/L sodium hydroxide at a flow rate of 0.3 mL/min. The amino acids quantification was performed using a fluorescence detector at wavelength excitation of 348 nm and emission at 450 nm.

2.7 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

The PPI, PPHP and PPHF (10 mg each) were mixed with 1.0 mL deionized water, which had 0.9 and 1.8 mg/ml of protein content using the Bradford method. In total 10 µg protein extract was mixed with 4 µL SDS sample buffer 4× (0.5 mol/L Tris HCl (pH 6.8)), glycerol 30% v/v, 8% w/v SDS, 0.5% bromophenol blue and dithiothreitol (DTT) 50 mmol/L. The protein samples were boiled in a sample buffer at 95°C for 10 mins and loaded. A stacking gel was prepared with 4% and 15% (v/v) polyacrylamide gel electrophoresis. A 20 µL sample solution was loaded on the Mini-Protein II cell apparatus (Atto Co., Tokyo, Japan) and run for 1 hr and 20 mins at 150 V. Protein bands were visualized after staining with Coomassie Brilliant Blue R250. The molecular weight range of proteins in the standard was from 11-245 kDa (Schagger and von Jagow, 1987).

2.8 In vitro antioxidant activities

In vitro antioxidant activities used spectrophotometric methods such as Arnao *et al*. (2001), Benzie and Strain (1996), Brand-Williams *et al*. (1995) and Thaipong *et al*. (2006). As much as 10 µL of samples were reacted with 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 10 mM 2,4,6-tri (2-pyridyl)-S-triazine 1,1-diphenyl-2-picrylhydrazyl, 20 mM $FeCl_3$ and 0.3 M acetate buffer, pH 3.6 mixed at a ratio of 1:1:5 (FRAP reagent) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) of 190 µL. Then the reaction mixture was incubated at room temperature in the dark for 30 mins (DPPH) and 15 mins (FRAP and ABTS assay) before the absorbance was read at 515 nm, 593 nm and 734 nm. The standard curve was linear between 25 and 600 mM Trolox. Results are expressed in mg Trolox equivalents (TEAC)/g sample.

2.9 Statistical analysis

The experiments were carried out in triplicates and data subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's multiple range tests with significance accepted at $p<0.05$.

3. Results and discussion

3.1 Physiochemical properties

The results of physiochemical properties are summarized in Table 1. In this work, the content of proteins of de-hulled pigeon pea presents at 19.58±0.07% and it was determined using the Dumas Combusion method. The yield of protein extract was 15.62% when compared to the weight of pigeon pea flour, while pigeon pea milling waste by-product had a higher yield (37%) of protein isolate (Tapal *et al*., 2019). The yield of protein isolated using alkaline (pH 9.0)-acid precipitation (pH 4.5) was about 64.42 g/100 g, which is higher than those obtained for Bambara groundnut protein isolate (56.08-58.70 g/100 g) (Adebowale *et al*., 2011). PPI presents a protein content of 80.78% of total component and PPHP presents a protein content of 73.53% of protein hydrolysates and 76.16% of protein after derived from flavourzyme hydrolysis for 4 hrs. Mwasaru *et al*. (1999) reported protein extraction (35-58%) from pigeon pea by the isoelectric point precipitation technique at the pH range of 8.5-12.5 with protein content in the isolate ranging from 78-83%. The authors also reported that the protein isolate prepared was free of trypsin inhibitor activity, phytate and oligosaccharides (Tapal *et al*., 2019). PPI and their hydrolysates present a low value of moisture with 1.0% and a low value of aw with 0.3 (Table 1). In this study, the protein content of PPI was higher than mature pigeon pea protein (76.41%), which was reported by Pazmiňo *et al*. (2018). This might be due to differences in the method of time, ratio and pH for isolation.

3.2 Degree of hydrolysis

Generally, the term DH is verified for indicating the percentage of cleaved peptide bonds, which is an important parameter for monitoring protein hydrolysis (Nielsen *et al*., 2001). The DH of PPHP at the end of the hydrolysis (4 hrs) was 59.62±2.13%, and DH at the same time of hydrolysis was obtained from flavourzyme hydrolysis (PPHF) was increased to 79.82±3.25%. The PPI was hydrolyzed with two enzymes in order to identify the enzymes that are able to cleave peptides with

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Table 1. Physiochemical properties of pigeon pea protein hydrolysates.

Physicochemical properties	PPI	PPHP	PPHF
%Yield	15.62	51.3	39.47
%Protein (N×6.25)	80.78±0.68[a]	73.53±0.13[c]	76.16±0.13[b]
%Recovery	64.42	75.44	60.13
%Moisture[ns]	1.06±0.10	1.04±0.02	1.02±0.01
Aw	0.30±0.00[b]	0.33±0.01[a]	0.35±0.03[a]
Color			
L	83.15±0.33[a]	80.64±0.31[b]	79.17±0.81[c]
a*	1.30±0.33[c]	1.92±0.04[b]	2.06±0.09[a]
b*	22.26±0.18[b]	20.94±0.48[c]	23.28±0.66[a]

Values are presented as mean±SD, n = 3. Values with different superscripts within the same row are statistically significantly different ($p<0.05$).
[ns] Not significant

high antioxidant activities. Pancreatin and flavourzyme were used in this work to produce PPHs with relatively moderate to high DH. The enzymes have broad specificity and act as endo and exo-endopeptidases that produce high-yielding peptides (Whitcomb and Lowe, 2007; Segura Campos *et al*., 2010; da Silva, 2017). It is worth noting that the mixture of endo-exo peptidases (pancreatin and flavourzyme) was able to cleave peptide bonds more efficiently when compared to the individual enzymes (Zhang *et al*., 2009). A similar DH result (70%) was also found in chickpea protein hydrolysis using alcalase and flavourzyme (Yust Mdel *et al*., 2012). In addition, the DH values observed in this work for the pancreatin enzyme hydrolysis are higher than the 27.7% for pigeon pea (Olagunju *et al*., 2018) and 24-38% for pigeon pea milling waste by-product (Tapal *et al*., 2019). However, the DH result (59.62%) is also close to the 58.84% for mung bean protein derived from 8 hrs of hydrolysis (Budseekoad *et al*., 2018). These results suggest the type of enzyme, their specificities and type of raw material, their protein composition, and amino acid sequence, play major roles in the ability of enzymes to cleave peptide bonds as indicated by DH results (Budseekoad *et al*., 2018).

3.3 Amino acid content

Legumes and seed are considered an alternative source of protein and amino acids, as a consequence the amino acid profile (g/100 g of sample) of PPI, PPHP and PPHF has therefore been determined (Table 2). A total of 18 amino acids were analyzed. The highest values of three samples were observed for glutamic acid (15.13-15.94 g/100 g), aspartic acid (8.45-8.84 g/100 g), leucine (6.05-6.48 g/100 g), phenylalanine (5.64-5.91 g/100 g), lysine (5.05-5.37 g/100 g), and serine (4.48-4.73 g /100 g), while the lowest values were for tryptophan (0.43-0.44 g/100 g). The present study revealed that pigeon pea protein and its hydrolysates are a protein-rich legume with a significant content of essential amino acids (EAA)

Table 2. Amino acid composition of pigeon pea protein isolate and its hydrolysates.

Amino acid	Pigeon pea protein hydrolysates (g/100 g sample)			Joint FAO/ WHO/UNU (2007)
	PPI	PPHP	PPHF	
Threonine	2.88	3.04	2.90	2.3
Methionine	1.07	1.06	1.01	1.6
Phenylalanine	5.64	5.91	5.72	3.8
Histidine	2.93	2.92	2.78	1.5
Lysine	5.36	5.37	5.05	4.5
Valine	2.48	3.11	3.03	3.9
Isoleucine	2.18	2.77	2.72	3.0
Leucine	6.05	6.48	6.19	5.9
Tryptophan	0.43	0.44	0.43	0.6
Cystine	0.77	0.75	0.68	
Serine	4.73	4.64	4.48	
Glycine	3.09	3.03	2.81	
Glutamic acid	15.94	15.73	15.13	
Proline	4.00	3.86	3.72	
Alanine	3.61	3.57	3.36	
Tyrosine	3.05	2.94	2.87	
Arginine	5.01	5.20	4.91	
Aspartic acid	8.73	8.84	8.45	
EAA	29.78	31.85	30.49	
NEAA	48.17	47.81	45.74	
HAA	29.28	30.90	29.72	
PCAA	8.28	8.28	7.83	
NCAA	24.68	24.57	23.58	
AAA	9.12	9.30	9.01	
SCAA	1.83	1.81	1.68	
BCAA	10.71	12.37	11.94	

EAA: essential amino acid, NEAA: non-essential amino acid, HAA: hydrophobic amino acids (alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine and cysteine), PCAA: positively charged amino acids (Histidine, lysine), NCAA: negatively charged amino acids (ASX(asparagine +aspartic acid) and GLX (glutamine+glutamic acid)), AAA: aromatic amino acids (phenylalanine, tryptophan and tyrosine), SCAA: sulphur containing amino acids (cysteine and methionine), BCAA: Branch chain amino acids (eucine, isoleucine and valine).

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exceeding the FAO/WHO/UNU Expert Consultation (2007) requirement for adults (Table 1). Glutamic and aspartic acids were the most prominent amino acids in the PPI, PPHP and PPHF but cysteine and tryptophan contents were below standard requirements. Proteolysis with pancreatin and flavourzyme slightly increased the content of branched-chain amino acids (BCAAs) by 15% and 11%. The highest levels of hydrophobic amino acids (alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine and cysteine) were also found with PPHP and PPHF. These results verify the specificity of pancreatin and flavourzyme (mixture of exo-endo peptidase) to cleave proteins at peptide bonds contributed by hydrophobic amino acids, thus, increasing the contents of the associated amino acids. Interestingly, increased compositions of hydrophobic amino acids (HAA) and aromatic amino acids (AAA) will encourage the lipid solubility of polypeptides, which could enhance antioxidant activity (Chalamaiah *et al*., 2012). The results displayed higher contents of aromatic amino acids 9.12% and 9.30% for PPI and PPHP, respectively when compared to earlier reports by Nwachukwu *et al*. (2014) for flaxseed protein and the thermoase hydrolysate (8.62 and 9.03% aromatic. The differences in amino acids found for the pigeon pea and their hydrolysates may be a consequence of the genetic variation, specific protease and hydrolysis time applied in commercial practices.

3.4 SDS-PAGE electrophoresis analysis of pigeon pea protein isolate and pigeon pea protein hydrolysate

Figure 1 shows the protein profiles present in PPI, PPHP (Lane 2) and PPHF (Lane 3). The band patterns of pigeon pea protein isolated are divided into multiple components with molecular weight (Mw) distribution ranging from 17 to 63 kDa, which originated mainly from vicilin and legumin. The pattern profile of PPI consisted of two major (63 and 48 kDa) and seven minor (17, 20, 23, 25, 27, 35 and 50 kDa) subunits of vicilin (7S globulin) protein from pigeon peas. Legumin was identified with two bands of basic (Mw 20 and 23 kDa) subunits (Barć *et al*., 2011; Pebrianti *et al*., 2019). PPI presents a protein profile more complex with more intensive bands. As shown in Figure 1, the change in the SDS-PAGE profile was detected after 4 hrs of digestion. PPHP was characterized by the presence of a new protein band of 8-10 kDa (4 hrs). The high intensity of the protein hydrolysis band was found with 48 kDa and 23 kDa while the protein band of 63 kDa disappeared. Considering among 3 PPHs fractions, PPHF found that the concentration of protein hydrolysates with lower Mw (20, 23 and 8 kDa) has increased due to broad cleavage obtained flavourzyme (exo-endo cleavage) during pigeon pea fermentation. Thus, this work confirms that large Mw of protein was derived into small Mw with bioactive properties by protease enzyme during the hydrolysis of pigeon PPI. In this work, results revealed that protein fractions with moderate to low Mw ranging from 8 to 48 kDa and containing high hydrophobic or aromatic amino acids could probably be associated with higher antioxidant activities. Therefore, the mechanism underlying the protective effect of protein isolated from pigeon peas as an antioxidant needs to be further explored.

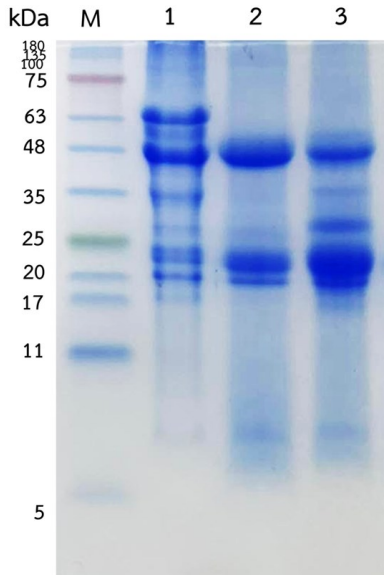

Figure 1. SDS-PAGE of the pigeon pea protein isolated shown in Lane 1. Lane 2: pancreatin-hydrolyzed pigeon pea protein (PPHP) and Lane 3: flavourzyme-hydrolyzed pigeon pea protein (PPHF) and Lane M" Mw standard marker (5-180 kDa).

3.5 Antioxidant activities

DPPH˙ is used to measure the ability of antioxidative compounds to donate electrons or hydrogen ions to free radicals to form a more stable compound. DPPH˙ receives hydrogen from antioxidants to form a stable diamagnetic molecule (yellow-colored diphenyl picrylhydrazyl), and the extent of discoloration of the radical from purple to yellow indicates the scavenging potential of the antioxidant compound with respect to its hydrogen-donating ability (Olagunju *et al*., 2018). Figure 2A shows the capacity of PPI and their hydrolysates to scavenge DPPH radical. PPI had the highest DPPH scavenging activity 98.40 mg TEAC/g sample when compared to PPHP (49.73 mg TEAC/g sample) and PPHF (56.84 mg TEAC/g sample. The result indicates that hydrophobic residues such as leucine, phenylalanine, and tryptophan were minor factors for DPPH scavenging activity considering among Mw distribution in 3 PPHs fraction, PPI exhibited that complication of polypeptides Mw could enhance the DPPH• - scavenging activity by increasing polypeptides holding while facilitating

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interaction and proton exchanges with radical species (Zou *et al*., 2016).

The results of $ABTS^{\cdot+}$ scavenging activity are presented in Figure 2B. There was a significant difference ($p<0.05$) among the scavenging activity of PPHF (671 mg TEAC/g sample), PPI (412 mg TEAC/g sample) and PPHP (624 mg TEAC/g sample). All the hydrolysates assessed showed higher scavenging $ABTS^{\cdot+}$ capability when compared to PPI. Similar to other results on the radical scavenging activity of pigeon pea protein (Olagunju *et al*., 2018), the protein isolate exhibited the lowest $ABTS^{+\bullet}$ scavenging activity. As shown in Table 2, relative to the amino acid composition of the isolate and hydrolysates, PPI had a high hydrophobic amino acid that high amount of HAA and AAA have been associated with low solubility which induces low ABTS radical scavenging activity (Zou *et al*., 2016). In the $ABTS^{+\bullet}$ method, the antioxidant activity was measured exclusively by the ability of an antioxidant to act as a hydrogen or electron donor to neutralize preformed $ABTS^{+\bullet}$ radicals (Re *et al*., 1999). The remarkable similarity in the results ABTS+• assays and DPPH assay on different hydrolyzes suggested that inhibition of radical initiation was probably not a critical factor determining the efficacy of PPHs digests as antioxidants (Ma *et al*., 2010).

Antioxidants donate hydrogen atoms to electron-deficient free radicals and this electron-donating ability can be evaluated using ferric-reducing antioxidant power (Olagunju *et al*., 2018). The antioxidant activity of a peptide directly correlates to its ferric ion-reducing ability. PPHF significantly increased the reducing power of the pigeon pea protein. The ability of PPHF to reduce Fe^{3+} was 35 mg TEAC/g sample and was compared to that of Trolox standard (20-600 mM) (Figure 2C). It was also observed that irrespective of the protease employed for enzymatic hydrolysis (pancreatin and flaovurzyme) and their individual specificities, the PPHP and PPHF acted as stronger (1.5-3.8 folds) reducing agents than the PPI. Similar to other results of the present study, Olagunju *et al*. (2018) reported the highest Fe^{3+} reducing ability for pigeon pea protein using pancreatin. Results of this work showed that low to medium molecular weight peptides (8, 20, 23, 48 kDa) exhibited stronger FRAP activity than the high molecular weight peptides, which correspond to other reports where >10 kDa also showed the strongest activity (Olagunju *et al*., 2018). The results from this work were in contrast to other reports where <1 kDa exhibited the highest activity (Ajibo *et al*., 2011; He *et al*., 2013; Chunkao *et al*., 2020). The results are consistent with literature reports indicating that the presence, sequence of various amino acids and molecular weight distribution are considered critical factors in the ability of peptides to scavenge free radicals.

4. Conclusion

This work showed that protein extraction from pigeon peas was strongly obtained, recovering most of the protein component by an alkaline environment (pH 9.0), followed by isoelectric precipitation at pH 4.5, increasing a protein yield 3-4 folds to initial protein. Regarding functional properties, the antioxidant activities seem suitable to scavenge a free radical for *in vitro* study, possibly due to the important increase in the content of hydrophobic amino acids. The peptide antioxidants were related to medium and high molecular weight (>10 kDa) of protein hydrolysates was found. The flavourzyme-hydrolyzed pigeon pea protein exhibited interesting polypeptides and had the strongest ABTS and FRAP capacity. These findings suggest that PPI and hydrolysates have the potential to be used in fortifying functional foods, which could enhance their nutritional and functional values.

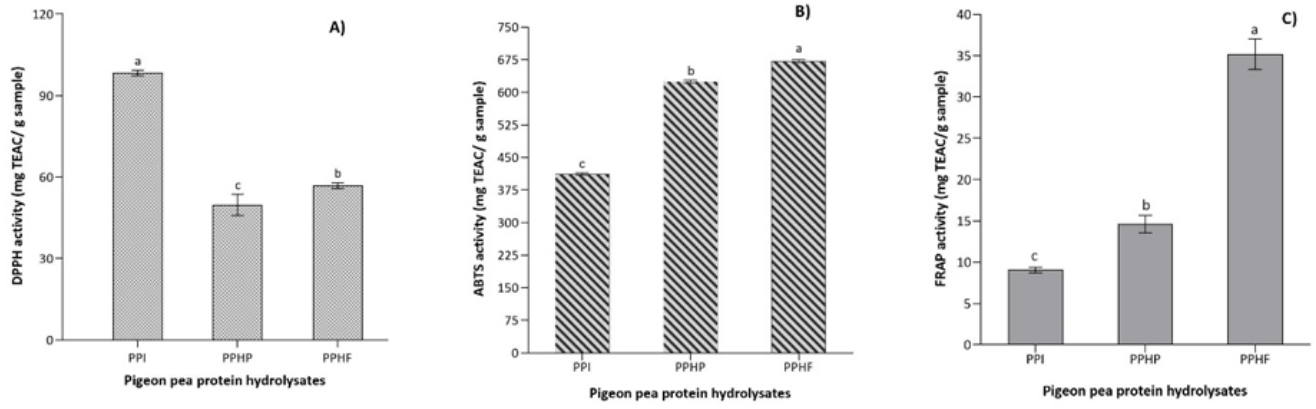

Figure 2. (A) DPPH activity, (B) ABTS activity and (C) FRAP activity of pigeon pea protein isolate (PPI), pancreatin-hydrolyzed pigeon pea protein (PPHP) and flavourzyme-hydrolyzed pigeon pea protein (PPHF). Bars with different notations are statistically significantly different ($p<0.05$).

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Conflict of interest

The authors declare no conflict of interest.

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