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Full Paper

Antioxidant activities of sacha inchi (*Plukenetia volubilis* L.) protein isolate and its hydrolysates produced with different proteases

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Abstract: Proteolytic enzymes pepsin, papain and Flavourzyme were used to produce sacha inchi protein hydrolysates SPHPe, SPHPa and SPHFl respectively. Pepsin gives significantly ($p< 0.05$) highest degree of hydrolysis (28.33%) and protein recovery (45.95%). The protein content of all protein hydrolysates is approximately 82-84%. However, SPHPe has the highest content of hydrophobic aliphatic amino acids (alanine, valine and proline) as well as hydrophobic aromatic amino acid (phenylalanine). SPHPe also shows the highest antioxidant activities by DPPH (55.7%), FRAP (0.167 mmol Fe^{2+}/g) and metal ion chelation (55.4%) assays while the sacha inchi protein isolate shows the highest hydroxyl radical scavenging activity (73.3%).

Keywords: sacha inchi, *Plukenetia volubilis*, protein isolate, protein hydrolysate, amino acid profile, antioxidant activity

INTRODUCTION

Protein hydrolysates have been used extensively for their nutritional, functional and bioactive properties. They comprise a complex mixture of oligopeptides, peptides and free amino acids produced from a partial or extensive protein hydrolysis process. Some types of protein hydrolysates have special biological properties such as antioxidant activity and anticancer properties

[1, 2]. Therefore, they are considered very beneficial and desirable in many fields. Diseases such as cancer, diabetes, arteriosclerosis, inflammatory disease, autoimmunity, cardiovascular disease and Alzheimer's have been associated with the increase in reactive oxygen species or the inability of the organism to reduce these active species that were normally produced by the organism cells, a process known as oxidative stress [3]. Antioxidants are important substances that have the ability to protect an organism from the damage caused by oxidative stress. Thus, there is special interest in the presence of natural antioxidants in medicinal plants as they may help an organism to maintain a normal balance of reactive oxygen species. Furthermore, these compounds have no negative effects on normal cells [3-5]. The characteristic peptide chemical structures may be the main factors that contribute to their antioxidant activities. These bioactive peptides may be released during in vivo digestion, in vitro enzymatic hydrolysis or food processing steps including fermentation [6]. It is well known that there are many factors involved in the antioxidant activity of food protein hydrolysates. One important factor is the specificity of the enzymes used for proteolysis [7] and the other is the degree of hydrolysis (DH) [8].

In 2017 around 271 acres of sacha inchi (*Plukenetia volubilis* L.) were cultivated in Thailand and about 80% of exported sacha inchi products comprised sacha inchi seed oil [9]. Sacha inchi seeds are normally used for the extraction of oil owing to their high oil content (~50%). The main by-product of oil production is the seed residue or meal, which contains more than 50% protein [10] and is normally discarded as waste or used as animal feed. The seed from this plant is an excellent source of essential amino acids such as cysteine, tyrosine, threonine and tryptophan [11]. According to Hamaker et al. [12], sacha inchi seeds contain leucine (64 mg/g), tyrosine (55 mg/g), isoleucine (50 mg/g), lysine (43 mg/g), valine (40 mg/g), sulphur amino acids; methionine+cysteine (37 mg/g) and phenylalanine (9 mg/g).

As per our knowledge, there is limited research work devoted to the exploitation of sacha inchi residue (a by-product of oil extraction) as a source of protein hydrolysate prepared from different proteases that include those from animal, microbe and plant. The protein hydrolysates produced with different proteases could display different functional bioactive properties especially antioxidant activities. The work of Chirinos et al. [13] reported obtaining protein hydrolysates from sacha inchi cake with interesting antioxidant properties using microbial enzymes (Alcalase, Neutrase and Flavourzyme). Rawdkuen et al. [14] reported that sacha inchi protein hydrolysates produced by crude papain and Calotropis proteases showed high antioxidant properties. The main objective of this study is to investigate the effects of different types of proteases (pepsin, papain and Flavourzyme) on the antioxidant activities of protein hydrolysates prepared from sacha inchi protein isolate (SIPI). All hydrolysate samples were also analysed for their degree of hydrolysis (DH) and amino acid composition compared with SIPI.

MATERIALS AND METHODS

Raw Material and Chemicals

Sacha inchi meal, a by-product of oil extraction, was kindly provided by Tai.C.M.S. Standard Industrial Co. (Chiang Rai province, Thailand). Three different protease enzymes were used, viz. pepsin, papain and Flavourzyme. Pepsin from porcine gastric mucosa (EC 3.4.23.1; ≥250 units/mg solid), papain from papaya latex (EC 3.4.22.2; 10 units/mg protein) and Flavourzyme® from *Aspergillus oryzae* (EC 232-752-2; ≥500 U/g) were purchased from Sigma-Aldrich (USA). Glutathione (GSH), glycyl-glycyl-glycine, 1,10-phenanthroline, ortho-phthalaldehyde, 2,2-

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diphenyl-1-picrylhydrazyl (DPPH) and 4,6-tripryridyl-s-triazine were purchased from Sigma-Aldrich (USA). Other chemical reagents were obtained from Fisher Scientific (Canada). All chemical reagents were of analytical grade and used without further purification, whereas distilled water was used for the preparation of reagents.

Production of Sacha Inchi Protein Isolate (SIPI)

SIPI was produced according to the method described by Adebowale et al. [15]. A 100 g of sacha inchi meal was dispersed in deionised water (1 L) and the mixture adjusted to pH 11.5 with 2M NaOH to solubilise proteins. The resultant dispersion was stirred at 60ºC for 1 hr and centrifuged (7000 x g, 30 min. at 4ºC). The supernatant was adjusted to pH 7.0 with 2M HCl to precipitate most of the proteins. Thereafter, the mixture was centrifuged (7000 x g, 30 min. at 4ºC) and the resultant precipitate was freeze-dried to produce SIPI.

Preparation of Sacha Inchi Protein Hydrolysates

Enzymatic hydrolysis of SIPI was carried out using protease enzymes (pepsin, papain or Flavourzyme). Briefly, a 100 g of the SIPI was stirred with 1000 mL of distilled water. The optimal pH of the solution was achieved using either 1M NaOH or 1M HCl. The optimal conditions for pepsin, papain and Flavourzyme were pH 2.0 at 37ºC, pH 7.0 at 55ºC and pH 6.5 at 50ºC respectively [16-18]. The hydrolysis process was performed by adding the protease enzyme to the protein solution at 1% w/w concentration (on the basis of protein content in the substrate). Once the enzyme was added, the temperature and pH were maintained and monitored for 4 hr as described above, after which hydrolysis was terminated by adjusting to pH 7.0 with either 1M NaOH or 1M HCl, followed by heating at 95ºC for 15 min. to ensure complete denaturation of the enzyme. The reaction mixture was then cooled to room temperature and centrifuged at 10000 x g for 30 min. at 4ºC using an Allegra^{TM} 6R centrifuge (Biotech Equipment Sales, USA) to separate the soluble hydrolysed materials (peptides) from the unhydrolysed residue (mainly undigested proteins). The clear supernatant was collected as the hydrolysate and a portion freeze-dried and stored at -20ºC until further analysis.

Determination of Degree of Hydrolysis (DH)

The o-phthalaldehyde (OPA) method described by Wanasundara et al. [19] was used to estimate DH with some modifications. The OPA reagent was freshly prepared by mixing 12.5 mL of 100 mM sodium tetraborate, 1.25 mL of 20% sodium tetraborate decahydrate, and OPA (20 mg) and 2-mercaptoethanol (50µL) dissolved in methanol (0.5 mL). A 200-µL aliquot of the OPA reagent was added to 10 µL of standard (glycyl-glycyl-glycine) or peptide samples and mixed. The mixture was incubated for 2 min. at room temperature and the absorbance was measured at 340 nm using a multiplate reader. The total number of primary amino groups was determined by acid hydrolysis of SIPI with 6 M HCl at 110ºC using an auto-digestion system (Gerhardt, Germany) for 24 hr. DH was defined as the percentage of cleaved peptide bonds as follows:

$$DH\ (\%) = [(NH_2)_{tx}-(NH_2)_{t0}/\ (NH_2)_{total}-(NH_2)_{t0}]\ x100(\%)\ ,$$

where $(NH_2)_{tx}$ = no. of free amino groups at X min., $(NH_2)_{total}$ = total no. of amino groups of SIPI and $(NH_2)_{t0}$ = amount of free amino groups at 0 min. of hydrolysis.

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Proximate Composition and Amino Acid Composition Analysis

The protein was determined using the Kjeldahl method [20]. Determinations of fat, ash, moisture content and total carbohydrate of SIPI and protein hydrolysates were carried out according to the official methods [20].

The amino acid composition of SIPI and protein hydrolysates were determined by HPLC (SpectraLab Scientific Inc, Canada) with a Pico-Tag column after hydrolysing the samples with 6 M HCl for 24 hr [21]. A separate digestion with performic acid was carried out in order to enable the determinations of methionine and cysteine [22], while tryptophan content was determined following hydrolysis with NaOH [23].

DPPH Radical Scavenging Assay

The scavenging activity of samples against the DPPH radical was determined using a previously described method [24] with some modifications for a 96-well clear flat-bottom plate. Samples were dissolved in 0.1 M sodium phosphate buffer (pH 7.0) containing 1% (w/v) Triton X-100. DPPH was dissolved in 95% methanol. A 100 µL of peptide samples at concentrations of 0.25, 0.5, 1.0 and 1.5 mg/mL were mixed with 100 µL of 0.1 mM DPPH solution in the 96-well plate. A blank well contained only DPPH and the sodium phosphate buffer. The plate was then covered and incubated in the dark at room temperature for 30 min. Thereafter, the absorbance of the sample and control was read at 517 nm. The scavenging activity of the samples was compared to that of GSH. The per cent scavenging activity of GSH and samples was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [(A_c - A_s)/ A_c] \times 100(\%) ,$$

where A_c = absorbance of control and A_s= absorbance of sample.

Hydroxyl Radical Scavenging Assay (HRSA)

The HRSA was modified based on a method described by Girgih et al. [25]. Briefly, each sample at 0.125, 0.25, 0.5 and 1.0 mg was mixed with 1 mL of 0.1M sodium phosphate buffer (pH 7.4). Fifty µL of 3 mM 1,10-phenanthroline in water and 50 µL 3 mM of $FeSO_4$ were added consecutively to 50 µL of sample, GSH or buffer (control) in a clear, flat bottom 96-well microplate. To initiate a reaction in the wells, 50 µL of 0.01% hydrogen peroxide solution were added to the mixture, which was then covered and incubated at 37^{o}C for 1 hr with shaking. Thereafter, the absorbance of the mixture was measured at 536 nm every 10 min. for a period of 1 hr. The $^{\bullet}$OH scavenging activity was calculated as described by Ajibola et al. [26], using the reaction rate (ΔA/min.) equation:

$$^{\bullet}\text{OH scavenging activity (\%)} = [(\Delta A/\text{min.})_{control} - (\Delta A/\text{min.})_{sample}) / (\Delta A/\text{min.})_{control}] \times 100(\%)$$

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP was determined according to the method of Benzie and Strain [27] with some modifications. Briefly, the FRAP reagent was freshly prepared by mixing 300 mM sodium acetate buffer (pH 3.6), 10 mM 4,6-tripryridyl-s-triazine in 40 mM HCl and 20 mM ferric chloride in the ratio 5:1:1 (v/v) before evaluation. Two hundred mL of the FRAP reagent (preheated to 37^{o}C) were added to 40 mL of samples (0.125, 0.25, 0.5 and 1.0 mg/mL) or GSH in a 96-well microplate. Absorbance at 593 nm was measured relative to a reagent blank. Ferrous sulphate (0.025-0.25 mM)

was used to prepare a standard curve and the results for the samples were expressed in mM $FeSO_4$. Increased absorbance of the reaction mixture indicates increased reducing power.

Determination of Fe^{2+} Chelating Activity

The iron-chelating activity determination was adapted from the method described by Ajibola et al. [26]. Five hundred uL of samples or GSH solutions (0.5, 1.0, 1.5 and 2.0 mg/mL) was mixed with 0.025 mL of 2 mM $FeCl_2$ and 0.925 mL distilled water in a reaction tube. Thereafter, 0.05 mL of 5 mM ferrozine solution was added and mixed thoroughly. The mixture was allowed to stand at room temperature for 10 min. and 200 µL aliquot of the reaction mixture was added to a clear-bottom 96-well microplate well. The absorbance of sample and blank was measured at 562 nm and the metal chelating activity of the sample was compared to that of GSH.

Statistical Analysis

Data were collected in triplicate and subjected to the analysis of variance (ANOVA) using SPSS 11.0 software. The significant differences among means were differentiated by Duncan's new multiple range tests at a statistical significance of 95%

RESULTS AND DISCUSSION

Degree of Hydrolysis (DH)

The progression of hydrolysis was monitored in terms of DH, which is presented in Figure 1. After 4 hr of reaction, the DH of the protein hydrolysates SPHPe, SPHPa and SPHFl (obtained by the use of pepsin, papain and Flavourzyme respectively) was 28.33%, 24.18% and 27.78% respectively. The difference in DH may be due to the difference in the structure and length of the peptides in protein hydrolysates, which causes the difference in the number of accessible peptide bonds [28]. The variation in DH obtained from different types of enzymes and substrates has been reported. Marinova et al. [29] reported that papain produced soya protein hydrolysates with a DH value of 23% while O'Keeffe and FitzGerald [30] stated that whey protein hydrolysate by Flavourzyme had 11.4% DH value. Amaranthus and buckwheat proteins were produced by pepsin with 11.6% and 17.0% DH respectively [31]. The difference in DH is related to the variation in the mechanism of peptide bond cleavage at different amino acid sequences and also to the difference in protein structure and/or composition [32].

Proximate Composition

Table 1 shows the proximate composition of SIPI and protein hydrolysates. The results show that the protein hydrolysates have lower protein contents than SIPI due to removal of insoluble protein by centrifugation. In addition, the fat contents of the protein hydrolysates are also reduced, which occurs from the centrifugation to separate the insoluble and undigested matter.

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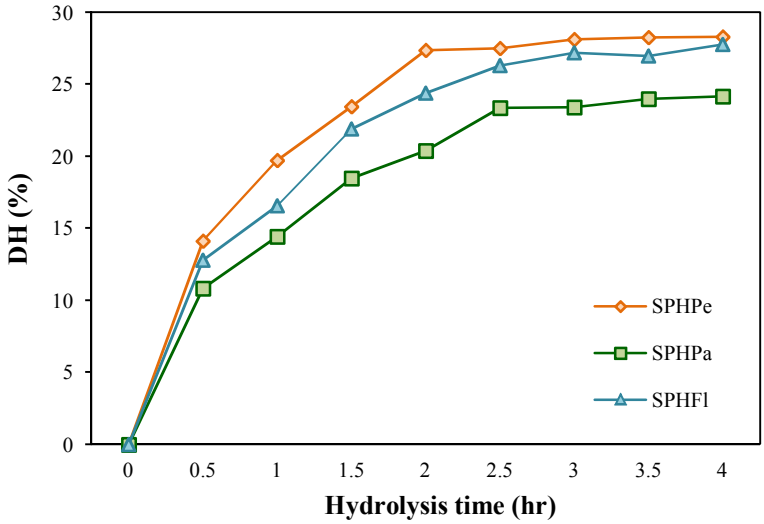

Figure 1. DH profile of sacha inchi protein hydrolysates

Table 1. Proximate composition and protein recovery of SIPI and protein hydrolysate samples

Samples	Protein/ peptide	Fat	Ash	Moisture	Carbohydrate
SIPI	89.35±0.05^{a}	3.33±0.16^{a}	1.16±0.12 ^{ns}	1.43±0.36^{ns}	4.73±0.05^{c}
SPHPe	84.37±0.05^{b}	2.03±0.01^{b}	1.24±0.23 ^{ns}	1.18±0.22^{ns}	11.18±0.11^{b}
SPHPa	82.52±0.07^{d}	2.11±0.07^{b}	1.09±0.11 ^{ns}	1.25±0.18^{ns}	13.03±0.08^{a}
SPHFl	83.63±0.11^{c}	2.10±0.19^{b}	1.71±0.04^{ns}	1.23±0.27^{ns}	11.33±0.03^{b}

$^{a\text{-}c}$ Mean values with different letters in the same column are significantly different at p <0.05.
^{ns} Mean values in the same column are not significantly different at p >0.05.

Amino Acid Composition

The amino acid composition study of nutraceutical materials is essential for understanding their nutritional values as well as functional and antioxidant properties [33]. The bioactive properties of proteins and peptides are highly influenced by their amino acid composition [34]. The amino acid compositions of SIPI and its hydrolysates are shown in Table 2. Slight differences are observed in the amino acid compositions of the protein hydrolysates when compared to SIPI. The content of hydrophobic aliphatic amino acids (alanine, valine and proline) as well as hydrophobic aromatic amino acid (phenylalanine) is highest in SPHPe. The hydrophobicity of peptides, which helps to improve their solubility in a lipid medium, has been reported to also improve their antioxidant potentials [35]. The low content of cysteine in SPHPa compared to SPHPe and SPHFl is probably due to the specificity of papain for peptide bonds involving mainly lysine or arginine and the adjacent amino acid residue. All sacha inchi protein hydrolysates in this study have higher contents of valine, aspartic acid, arginine, tyrosine and cysteine, in comparison with sesame, hen's egg, soy and pea protein hydrolysates [36]. According to Malomo and Aluko [17], the bioactive properties of a peptide are greatly influenced by its amino acid composition.

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Table 2. Amino acid compositions (%) of SIPI and its protein hydrolysate

Amino acid	SIPI	SPHPe	SPHPa	SPHFl
Aspartic acid	7.61	10.51	7.94	10.04
Threonine*	3.74	4.15	3.94	3.53
Serine	3.40	5.06	3.94	5.31
Glutamic acid	9.09	11.46	10.69	10.76
Proline	2.76	3.57	3.07	3.22
Glycine	3.42	4.08	3.71	4.25
Alanine	2.15	3.60	2.72	3.09
Cysteine	1.10	1.14	1.04	1.16
Valine*	3.06	4.69	3.17	4.16
Methionine*	0.53	0.78	0.78	1.01
Isoleucine*	2.10	3.71	3.12	3.16
Leucine*	3.31	5.91	4.31	5.12
Tyrosine	2.50	4.15	2.68	3.43
Phenylalanine*	1.02	1.84	1.26	1.35
Histidine*	1.11	2.31	1.32	2.06
Lysine*	2.20	4.05	3.03	3.57
Arginine	6.92	8.15	7.27	8.14
Tryptophan*	0.96	2.39	1.07	1.55

* Essential amino acid

DPPH Radical Scavenging Assay

The result of DPPH scavenging activities of SIPI and its protein hydrolysates is shown in Figure 2. The radical scavenging activities of all protein hydrolysates are much higher than those of SIPI but lower than those of GSH, which was used as reference. The DPPH scavenging activities of SIPI and all hydrolysates are concentration-dependent and reach 7.35%, 36.48%, 43.22% and 55.70% for SIPI, SPHPa, SPHFl and SPHPe respectively at 1 mg/mL. This indicates that the different protease enzymes produce products with different improved inhibitory potentials against free radicals compared to the precursor protein isolate. The improved radical scavenging potential of the three hydrolysates correlates with their DH level. Some amino acids, especially tyrosine, methionine, histidine and tryptophan, have also been shown to play specific roles in improving the antioxidant properties of peptides [37]. The hydrolysate samples in this study show better inhibitory potential than that of Moringa oleifera seed [38] and kidney bean [39] hydrolysates.

Hydroxyl Radical Scavenging Activity (HRSA)

The hydroxyl radical can be generated by a biochemical reaction: superoxide radical is converted by superoxide dismutase to hydrogen peroxide which can subsequently produce the extremely reactive hydroxyl radical in the presence of a divalent metal ion such as iron and copper [40]. This can lead to the oxidation of virtually all organic cell constituents including proteins [40]. Thus, hydroxyl radical scavenging is imperative for protection against various oxidative stress-induced diseases [41]. The HRSA of SIPI and its protein hydrolysates at different concentrations (0.125-1.0 mg/mL) was investigated. As illustrated in Figure 3, all samples show increased HRSA

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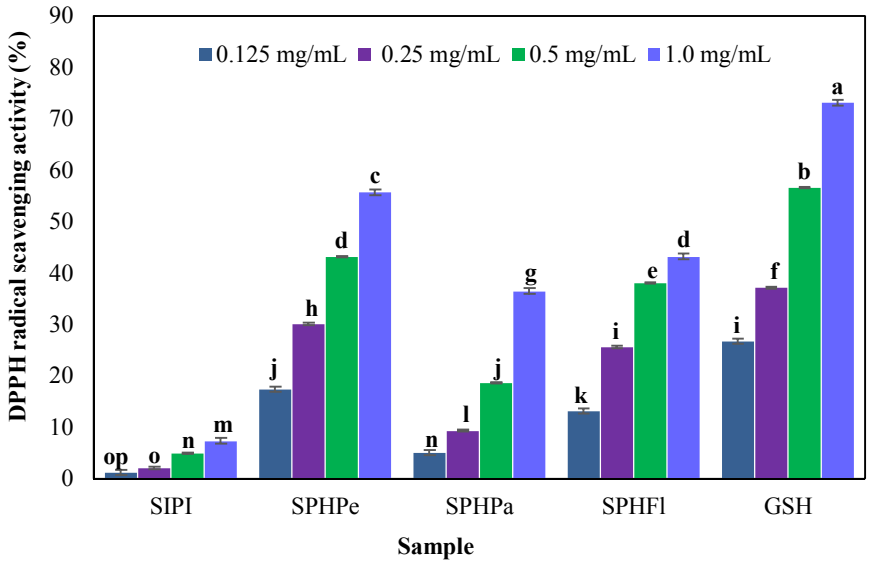

Figure 2. DPPH radical scavenging activity (%) of SIPI and its hydrolysates at different concentrations in comparison with GSH. Results are presented as mean ± standard deviation (n=3). Bars with different letters have mean values that are significantly different at $p < 0.05$.

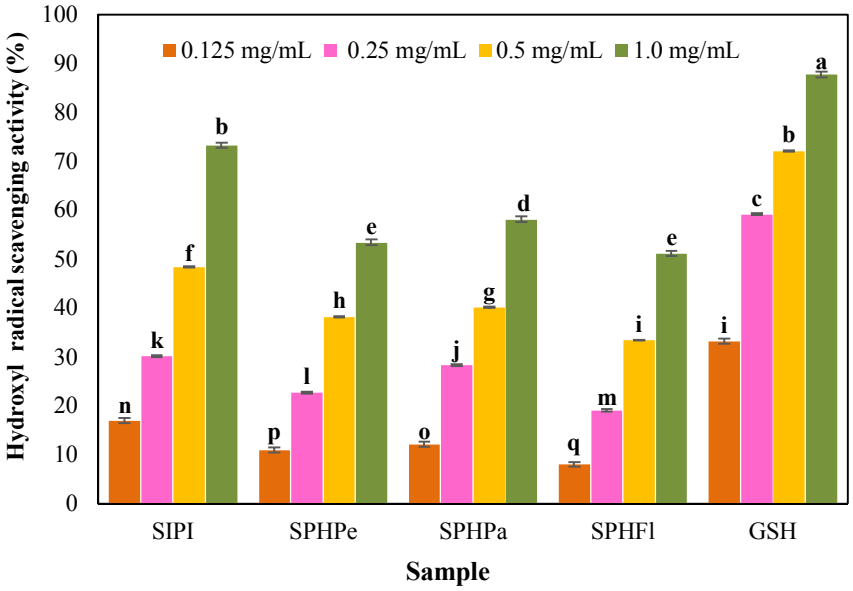

Figure 3. HRSA of SIPI and its hydrolysates at different concentrations in comparison with GSH. Results are presented as mean ± standard deviation (n=3). Bars with different letters have mean values that are significantly different at $p < 0.05$.

with increasing concentrations. Among the all samples, SIPI exhibits the highest radical scavenging activity (73.26% at 1.0 mg/mL) followed by SPHPa (58.11% at 1.0 mg/mL), while the lowest HRSA is obtained with SPHFl (8.04 % at 0.125 mg/mL) ($p < 0.05$). For SPHPe, the hydroxyl radical scavenging activities at 0.125 mg/mL is 10.98% and increases to 53.41% at 1.0 mg/mL. Thus, the protein isolate seems to provide better protection for cells against damage by hydroxyl radicals when compared to the hydrolysates. The higher HRSA of SIPI might come from the synergistic effect of the proteins and other bioactive compounds such as tocopherols and other phenolic compounds present in the un-hydrolysed sample [11]. The results obtained in this study are

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similar to those reported for hemp protein hydrolysate [25], kidney bean protein hydrolysate [39] and Moringa oleifera seed [38].

Ferric Reducing Antioxidant Power (FRAP)

FRAP evaluates the electron donating potential of antioxidant compounds, whereby the Fe^{3+}/ferricyanide complex is reduced to the ferrous form [40]. The results of the FRAP by SIPI and its protein hydrolysates at concentrations of 0.125, 0.25, 0.5 and 1.0 mg/mL are shown in Figure 4.

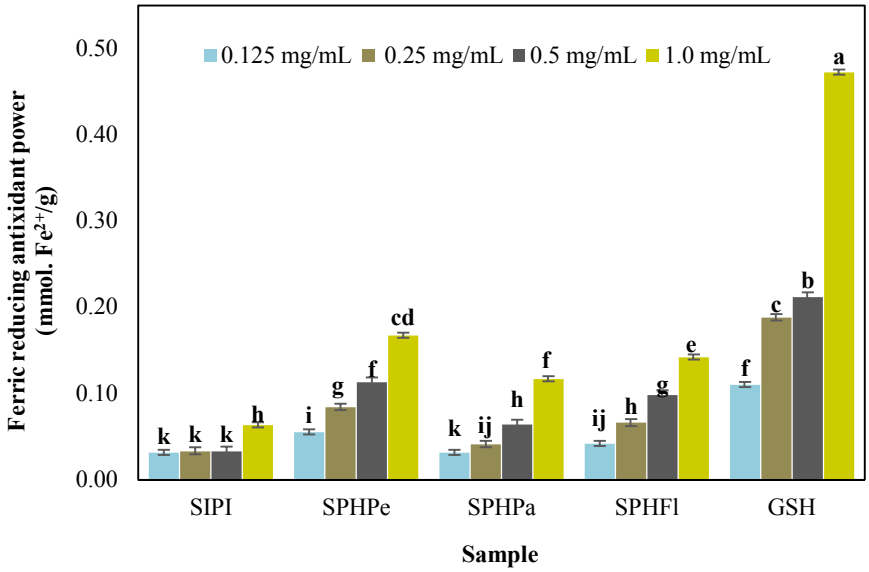

Figure 4. FRAP of SIPI and its hydrolysates at different concentrations in comparison with GSH. Results are presented as mean ± standard deviation (n=3). Bars with different letters have mean values that are significantly different at $p < 0.05$.

The GSH at concentration of 0.25 mg/mL shows a greater reducing power (0.188 mmol Fe^{2+}/g) than that of SIPI and all protein hydrolysates at 1.0 mg/mL concentration, which suggests that SIPI and its hydrolysates are poor reducing agents with respect to electron donation to ferric ion, with SPHPe showing the highest FRAP at 0.167 mmol Fe^{2+}/g. The FRAP of the protein hydrolysates also seems to correlate with their DH, which depends on the type of enzyme. It is a fact that the mechanisms of action of the antioxidants in FRAP and DPPH assays are similar [41]. Antioxidants in both assays react by the hydrogen atom transfer, and the redox potential of Fe^{3+}/ferricyanide complex and DPPH is comparable. According to Wang and Xiong [42], the strong reducing power of the protein hydrolysates could be the result of an increase in the availability of hydrogen atoms from the cleavage of the peptide bonds. In addition, amino acids with the strongest reducing powers tend to be those containing nucleophilic sulphur-containing side chains (cysteine and methionine) or aromatic side chains (tryptophan, tyrosine and phenylalanine) [43].

Fe^{2+} Chelating Activity

The highly reactive hydroxyl radical can be generated from the redox-active Fe(II) when it comes in contact with hydrogen peroxide through the Fenton reaction. The production of a high level of hydroxyl radicals may lead to the onset of various oxidant-induced metabolic disorders. This may explain the critical link between the level of Fe(II) and oxidative stress in humans [39]. The Fe^{2+} chelating activities of SIPI and its protein hydrolysates at concentrations of 0.5, 1.0, 1.5

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and 2.0 mg/mL are shown in Figure 5. The protein hydrolysates display significantly stronger Fe^{2+} chelating activities than those of GSH while SIPI shows the lowest activity. Jamdar et al. [44] have reported that a higher DH, leading to a sample with constituents of lower molecular weights, results in an increased metal ion chelating capacity. In addition, the presence of some side groups, especially of the basic amino acids lysine, histidine and arginine in the peptides, have been reported to be involved in chelating metal ions [45].

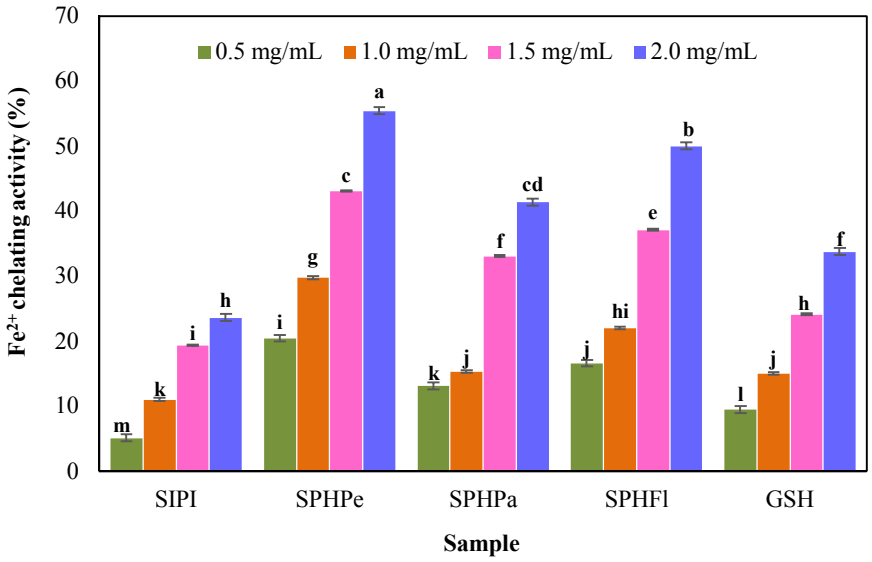

Figure 5. Fe^{2+} chelating activity of SIPI and its hydrolysates at different concentrations in comparison with GSH. Results are presented as mean ± standard deviation (n=3). Bars with different letters have mean values that are significantly different at $p < 0.05$.

CONCLUSIONS

Enzymatic hydrolysis of SIPI by pepsin, papain and Flavourzyme seems to release peptides with a greater antioxidant properties. With the highest DH and a high content of hydrophobic amino acids, the SPHPe hydrolysate obtained with pepsin exhibits significantly highest antioxidant acitivities by DPPH, FRAP and Fe^{2+} chelation methods. For HRSA, however, SIPI exhibits the highest values. Further study involving peptide purification and *in vivo* experiments are needed to confirm the *in vitro* antioxidant properties and potential health benefits.

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