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ARTICLE

Amino Acid Composition and Antioxidant Properties of Pea Seed (*Pisum sativum* L.) Enzymatic Protein Hydrolysate Fractions

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The amino acid composition and antioxidant activities of peptide fractions obtained from HPLC separation of a pea protein hydrolysate (PPH) were studied. Thermolysin hydrolysis of pea protein isolate and ultrafiltration (3 kDa molecular weight cutoff membrane) yielded a PPH that was separated into five fractions (F1−F5) on a C_{18} reverse phase HPLC column. The fractions that eluted later from the column (F3−F5) contained higher contents hydrophobic and aromatic amino acids when compared to fractions that eluted early or the original PPH. Fractions F3−F5 also exhibited the strongest radical scavenging and metal chelating activities; however, hydrophobic character did not seem to contribute to reducing power of the peptides. In comparison to glutathione, the peptide fractions had significantly higher ($p < 0.05$) ability to inhibit linoleic acid oxidation and chelate metals. In contrast, glutathione had significantly higher ($p < 0.05$) free radical scavenging properties than the peptide fractions.

KEYWORDS: Pea protein hydrolysate; antioxidant properties; amino acid composition; thermolysin; ultrafiltration; reverse-phase HPLC; glutathione

INTRODUCTION

Yellow field pea seed is an important global commodity, and Canada is the world's largest producer and exporter (*1*), with an annual production of 30% of the world's dry pea stock in 2007−2008 (*2*). Pea seed is considered to be a significant source of protein, consisting of approximately 25% protein content by weight (*3*). Pea proteins have an amino acid profile comparable to that of other commonly consumed legumes (*3*) and contain a negligible amount of sulfur-containing amino acids (*4*). Pulse-derived peptides are generating interest for the production of bioactive peptides because they are more cost-effective in comparison to animal proteins (*5*).

Bioactive peptides commonly contain 3−20 amino acids per peptide as inactive sequences within large proteins and are released when the parent protein is hydrolyzed by digestive enzymes (in vitro and in vivo), by microbial enzymes, or during food processing (*6*). Enzymatic hydrolysis of proteins is one approach used to release bioactive peptides and is widely applied to improve functional and nutritional properties of protein sources (*7*). The biological activity of a peptide is widely recognized to be based on amino acid composition (*6*). Peptides could be used in the formulation of functional foods and nutraceuticals to prevent damage related to oxidative stress in human disease conditions. Moreover, natural antioxidants are desirable because they can be used at higher concentrations without the toxic side effects associated with the use of synthetic equivalents (*5*, *8*).

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Extensive research exploring the antioxidant activity of peptides hydrolyzed from food proteins has been conducted; however, the structure−function relationship between peptide characteristics and antioxidant properties has not been fully elucidated (*7*). Therefore, the objective of this study was to determine the effects of hydrophobic character and amino acid composition on the antioxidant activities of low molecular weight (LMW) peptide fractions obtained from reverse-phase (RP) HPLC separation of an enzymatic pea protein hydrolysate.

MATERIALS AND METHODS

Production of LMW Peptides from Pea Protein Isolate. Pea protein isolate (80% protein, dry weight basis) was a gift from Nutri-Pea Ltd. (Portage La Prairie, MB, Canada). Pea protein isolate was dispersed in distilled water to obtain 6.0% (w/v) protein slurry. Under stirring with a magnetic stirrer, the slurry was heated to 55 °C and adjusted to pH 8.0 using dilute NaOH solution. Thermolysin (Sigma Chemicals, St. Louis, MO) was added to initiate hydrolysis at a ratio of 0.5% (on the basis of protein weight, w/w). The temperature and pH of the slurry were maintained constant for 3 h, after which the hydrolysis was stopped by heating the slurry to 95 °C and held for 15 min. The hydrolysate was cooled to room temperature and centrifuged at 10000*g* for 25 min at 4 °C. The clear supernatant was collected and passed through a stirred ultrafiltration cell using a 3 kDa molecular weight (MW) cutoff membrane (Sartorius Co., Germany). The resulting permeate containing peptides with MW < 3 kDa was collected, freeze-dried, and stored at −20 °C for further use.

RP-HPLC Separation of Pea Peptides. Freeze-dried protein hydrolysate (< 3 kDa permeate) was dissolved in 0.1% trifluoroacetic acid in double-distilled water (solvent A) at a concentration of 100 mg/mL, and 2 mL (filtered through 0.2 μm membrane disk) was injected into a high-performance liquid chromatography (HPLC) system (Varian 940-LC)

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fitted with a Phenomenex C12 preparative column (21 × 250 mm). The sample was eluted from the column at a flow rate of 5 mL/min using a linear gradient of 0% of 0.1% trifluoroacetic acid in methanol (solvent B) to 100% B over 60 min; peptide elution was monitored as absorbance at 214 nm. Fractions were collected using an automated fraction collector every 30 s and pooled into five fractions according to their time of elution from the column. The pooled fractions were freeze-dried (after solvent evaporation) and stored at −20 °C until further use.

Amino Acid Analysis. An HPLC system was used to determine the amino acid profiles after samples were hydrolyzed with 6 M HCl according to the method given in ref *9*. The cysteine and methionine contents were determined after performic acid oxidation (*10*), and tryptophan content was determined after alkaline hydrolysis (*11*).

DPPH• Scavenging Assay. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay was carried out as described in ref *12* with minor modifications. Peptide samples were dissolved in 0.1 M sodium phosphate buffer, pH 7.0, containing 1% (w/v) Triton X-100. DPPH was dissolved in methanol to a final concentration of 100 μM. A blank control was run with samples consisting of DPPH and sodium phosphate buffer, whereas the standard consisted of glutathione (final concentration of 1 mg/mL) dissolved in phosphate buffer and mixed with DPPH solution. Peptide samples or glutathione (100 μL) at a final concentration of 1 mg/mL were mixed with 100 μL of the DPPH solution. The experimental solution stood at room temperature in darkness for 30 min, and then the absorbance was read at 517 nm ($A_{517\ nm}$). DPPH radical scavenging activity (%) was calculated as $\{[(A_{517\ nm})_c - (A_{517\ nm})_s]/(A_{517\ nm})_c\} \times 100$, where c and s represent blank control and sample, respectively.

Reducing Power. The reducing power of pea peptide fractions was measured according to previous methods (*13*, *14*) with slight modifications. An aliquot (250 μL in 0.2 M phosphate buffer, pH 6.6) of the peptide samples (or glutathione), at a final concentration of 1 mg/mL, was added to 250 μL of the phosphate buffer and 250 μL of 1% potassium ferricyanide solution. The solutions were mixed and heated at 50 °C for 20 min. After incubation, 250 μL of 10% trichloroacetic acid (TCA) was added, and 250 μL of the resulting mixture was combined with 50 μL of 0.1% ferric chloride and 200 μL of distilled water. After 10 min of incubation at room temperature, the solution was centrifuged at 10000*g*. The supernatant was collected into a 96-well microplate and absorbance of the supernatant measured at 700 nm. Strong reducing power of a sample is indicated by an increase in absorbance (*14*).

Superoxide Scavenging Activity. The superoxide scavenging activity of the pea peptides was measured according to our previous method (*15*). An aliquot of peptide samples or glutathione (80 μL in 50 mM Tris-HCl buffer containing 1 mM EDTA, pH 8.3, at a final concentration of 1 mg/mL) was mixed with 80 μL of the buffer directly into a clear bottom 96-well plate in darkness. Then, 40 μL of 1.5 mM pyrogallol dissolved in 10 mM HCl was added to each well. The reaction rate (Δ*A*/min) was measured immediately at 420 nm for 4 min at room temperature using the buffer as a control. The superoxide scavenging activity was calculated using the following equation:

$$\text{superoxide scavenging activity (\%)} = \{[(\Delta A/\text{min})_c - (\Delta A/\text{min})_s]/(\Delta A/\text{min})_c\} \times 100$$

Hydrogen Peroxide (H_2O_2) Scavenging Activity. The H_2O_2 scavenging activity assay method is based on a method developed by Guo et al. (*16*), with modifications. An aliquot (60 μL) of 0.1 mM aqueous solution of H_2O_2 was combined with 2.19 mL of 50 mM sodium phosphate buffer, pH 7.0, 300 μL of peptide fraction or glutathione (final concentration of 1 mg/mL dissolved in phosphate buffer), 30 μL of 9.7 U/mL peroxidase (in cold phosphate buffer), and 15 μL of 1 mM scopoletin (in methanol). The solution was mixed vigorously, and an aliquot of 200 μL was immediately removed and placed in a quartz cuvette. The change in fluorescence intensity (ΔFI) was measured for 60 s using an excitation wavelength of 366 nm and an emission wavelength of 460 nm with slit width of 2.5 nm in a Jasco FP-6300 spectrofluorimeter (Japan Spectroscopic Co., Tokyo, Japan) equipped with a thermostated cell compartment that was maintained at 37 °C with a circulatory water bath. A control blank was run by replacing the peptide sample volume with 300 μL of sodium phosphate buffer. The percent H_2O_2 scavenging activity was calculated using the following equation:hydrogen peroxide scavenging (%) = $[(\Delta FI_{control} - \Delta FI_{sample})/\Delta FI_{control}] \times 100$.

Metal Chelating Assay. The metal chelating assay was based on a previous method (*17*) with slight modifications. An aliquot (1 mL) of aqueous peptide sample or glutathione at a final concentration of 1 mg/mL was combined with 0.05 mL of $FeCl_2$ solution (2 mM) and 1.85 mL of double-distilled water. FerroZine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt) solution (0.1 mL, 5 mM) was added and mixed vigorously. The mixture stood at room temperature for 10 min followed by the addition of 200 μL into a clear-bottom 96-well microplate, and absorbance was measured at 562 nm. In the control, the peptide sample was replaced with double-distilled water. The chelating effect was calculated by the following equation:

$$\text{chelating effect (\%)} = \{[(A_{562nm})_{control} - (A_{562nm})_{sample}]/(A_{562nm})_{control}\} \times 100$$

Hydroxyl Radical (OH•) Scavenging Assay. The OH• scavenging assay was modified on the basis of a method described by Li et al. (*8*). Peptide samples or glutathione (50 μL at a final concentration of 1 mg/mL in 0.1 M sodium phosphate buffer, pH 7.4) were first added to a 96-well microplate followed by the addition of 50 μL of 3 mM 1,10-phenanthroline (in phosphate buffer) and 50 μL of 3 mM $FeSO_4$ (in water). To initiate the reaction, 50 μL of 0.01% aqueous H_2O_2 was added, and the reaction mixture was covered and incubated at 37 °C for 1 h with shaking. The absorbance was measured at 536 nm using a spectrophotometer. The absorbance was also determined for a blank (without peptide and H_2O_2) and a control (without peptide). The OH• scavenging activity was calculated as described by Li et al. (*8*).

Inhibition of Linoleic Acid Oxidation. Linoleic acid oxidation was measured according to a method described by Li et al. (*8*). Peptide samples or glutathione were dissolved in 1.5 mL of 0.1 M phosphate buffer, pH 7.0, at a final concentration of 1 mg/mL. The mixture was added to 1 mL of 50 mM ethanolic linoleic acid and stored in a glass test tube that was kept at 60 °C in darkness for 7 days. At 24 h intervals, 100 μL of the sample solution was mixed with 4.7 mL of 75% aqueous ethanol, 0.1 mL of ammonium thiocyanate (30% w/v), and 0.1 mL of 0.02 M ferrous chloride dissolved in 1 M HCl. The degree of color development was measured as increase in absorbance at 500 nm after 3 min of incubation at room temperature. An increase in absorbance indicates an increase in linoleic acid oxidation.

Statistical Analysis. All results are presented as means ± standard deviation from triplicate analysis. Statistical analysis was performed with SAS (Statistical Analysis Software 9.1) using one-way ANOVA. Duncan's multiple-range test was carried out to compare means between peptide fractions. Results are considered to be significant at $p < 0.05$. Correlation coefficients were calculated using GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA).

RESULTS AND DISCUSSION

Pea Protein Hydrolysates. Enzymatic hydrolysis of food proteins has been shown to liberate peptides with various bioactive properties. The amino acid compositions of the pea protein isolate (PPI) and <3 kDa pea protein hydrolysate (PPH) are presented in **Table 1**. Hydrolysis of PPI with thermolysin and subsequent ultrafiltration yielded a LMW PPH fraction with a protein content of 86% and slightly increased concentration of hydrophobic amino acids. In particular, there was a marked increase of branched-chain amino acids (isoleucine and leucine) and phenylalanine (**Table 1**). It is expected that upon hydrolysis with thermolysin, an increase in hydrophobic amino acids appear in the hydrolysate as the enzyme hydrolyzes from the amino end of the peptide bond at hydrophobic residues (*18*). In contrast, the positively charged amino acids (arginine, lysine, and histidine) decreased in concentration after hydrolysis and ultrafiltration (**Table 1**). Because thermolysin specifically cleaves hydrophobic amino acids, it could not have released a lot of peptides containing cationic amino acids, and this resulted in their decreased amounts in the protein hydrolysate.

Table 1. Amino Acid Composition of Pea Protein Isolate (PPI), <3 kDa Pea Protein Hydrolysate (PPH), and HPLC Fractions (F1−F5)

amino acid[a]	PPI (%)	PPH (%)	F1 (%)	F2 (%)	F3 (%)	F4 (%)	F5 (%)
Asx	11.81	13.79	13.94	10.63	12.59	10.85	11.04
Thr	3.48	3.6	3.89	3.86	3.34	3.11	3.22
Ser	5.72	6.2	6.63	5.71	6.19	4.41	3.82
Glx	16.54	13.92	17.12	14.78	13.75	12.87	6.64
Pro	5.49	5.15	2.33	6.47	5.14	5.42	8.05
Gly	4.09	3.76	3.52	5.00	3.96	4.66	3.26
Ala	4.34	5.01	5.54	4.30	5.03	3.44	3.62
Cys	0.87	0.24	0.18	0.39	0.39	0.38	0.29
Val	5.19	5.63	5.23	4.45	4.13	5.82	7.68
Met	1.12	0.91	0.70	1.70	0.87	1.07	0.68
Ile	4.73	5.43	4.13	4.04	6.71	5.85	9.13
Leu	8.79	9.91	8.70	6.68	9.95	14.57	19.48
Tyr	3.78	3.87	2.77	5.33	7.15	5.09	2.44
Phe	5.49	7.41	3.97	7.76	8.73	12.03	16.44
His	1.74	1.61	2.49	3.28	1.90	1.81	0.63
Lys	7.35	6.1	9.07	7.35	4.26	3.31	1.20
Arg	8.6	6.83	9.79	8.00	5.15	3.97	1.22
Trp	0.83	0.68	0.00	0.27	0.74	1.36	1.16
HAA	40.63	44.24	33.56	41.39	48.85	55.01	68.97
PCAA	17.69	14.54	21.35	18.63	11.32	9.08	3.05
NCAA	28.35	27.71	31.06	25.41	26.34	23.72	17.68
AAA	10.1	11.96	6.74	13.36	16.62	18.48	20.03

[a] Asx, aspartic acid and asparagine; Glx, glutamic acid and glutamine; combined total of hydrophobic amino acids (HAA) = alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine, and cysteine; positively charged amino acids (PCAA) = arginine, histidine, lysine; negatively charged amino acids (NCAA) = Asx and Glx; aromatic amino acids (AAA) = phenylalanine, tryptophan, and tyrosine.

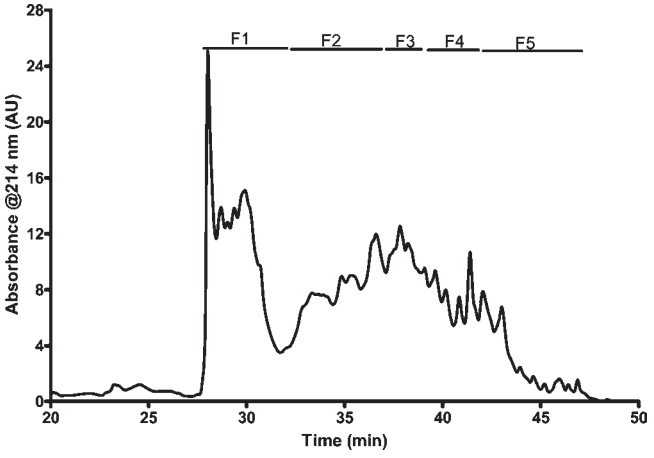

Figure 1. Fractionation of pea protein hydrolysate (<3 kDa permeate) by RP-HPLC using a C12 column. F1−F5 refer to pooled peptide fractions, which were collected, freeze-dried, and used for various determinations.

HPLC Fractionation. RP-HPLC separates peptides on the basis of their differences in hydrophobic properties (*19*). As shown in **Figure 1**, fractionation of the PPH on a preparative Phenomenex C12 column was pooled into five peptide fractions (F1−F5). F1 eluted first at 28 min and has the least net hydrophobicity of fractions eluting later because it was bound weakly to the column. F5 was bound strongly to the column and eluted last and, therefore, has the strongest net hydrophobic properties in comparison to the earlier eluting fractions. The protein yields (% of total recovered peptides) of the RP-HPLC fractions were 39.97% (F1), 24.63% (F2), 14.56% (F3), 12.92% (F4), and 7.91% (F5).

Amino Acid Composition of HPLC Fractions. As shown in **Table 1**, hydrophobic aliphatic amino acids (valine, leucine, and isoleucine) increased in percentage as the retention time of the fractions increased, especially for F4 and F5. However, alanine decreased in concentration with increasing retention time of the fractions. There was a >2-fold increase in the percentage of leucine and isoleucine in F5 (19 and 9%, respectively) in comparison to F1 (8.7 and 4%, respectively). The hydrophobic aromatic amino acids, tryptophan and phenylalanine, also increased from F1 to F5. Tryptophan was not detected in F1 and increased to approximately 1.2% in F5. The percentage of phenylalanine increased 4-fold from F1 to F5. The percentage of tyrosine was highest in F3 and lowest in F1 and F5. Proline, a slightly hydrophobic amino acid, increased approximately 3.5 times from F1 to F5. Moreover, there was a linear decrease of hydrophilic amino acids (serine, lysine, arginine, and histidine) from F1 to F5. Asparagine/aspartic acid and glutamine/glutamic acid also decreased from F1 (14 and 17%, respectively) to F5 (11 and 6.6%, respectively). Thus, the fractionation of the pea peptides using a hydrophobic HPLC column resulted in increased concentration of hydrophobic amino acids as retention time increased.

Radical Scavenging Activity. DPPH$^{\bullet}$ is not a biologically relevant radical; however, it is widely used to evaluate the antioxidant activity of natural compounds (*15*, *20*). The DPPH$^{\bullet}$ scavenging activity indicates the ability of the antioxidant compound to donate electrons or hydrogen, thereby converting the radical to a more stable species (*21*). As shown in **Figure 2A**, the fractions that eluted late (strongest hydrophobic character) possessed the strongest DPPH$^{\bullet}$ scavenging activity, whereas early eluting fractions had less activity. Thus, F4 and F5 showed the strongest activity in comparison to F1−F3. The radical scavenging activity of F5 is almost twice as high as that of F2. Moreover, F4 and F5 have similar DPPH$^{\bullet}$ scavenging activity as the PPH, which indicates that these two fractions contributed to the majority of the DPPH scavenging activity observed for the PPH. Amino acid analysis showed that F4 and F5 are both rich in hydrophobic amino acids including leucine, phenylalanine, valine, and tryptophan compared to F1−F3. Tryptophan has been reported to play an important role in the DPPH radical scavenging activity of purified patatin, perhaps as a hydrogen donor, because its inactivation via chemical modification resulted in reduced antioxidant activity of the protein (*22*). In this study, glutathione possessed the strongest DPPH$^{\bullet}$ scavenging activity (**Figure 2A**). Glutathione is recognized to be a potent antioxidant, and the activity is attributed to the sulfhydryl group of cysteine; therefore, cysteine-containing peptides could be considered as effective scavengers of DPPH$^{\bullet}$. However, because F5 contained a low amount of cysteine, a combination of other amino acids enriched in this fraction (leucine, phenylalanine, valine, and tryptophan) may have contributed to its DPPH$^{\bullet}$-scavenging activity. This is supported by the positive correlation (R^2 = 0.83) between the DPPH$^{\bullet}$ scavenging activity and the total amount of hydrophobic amino acids in the peptide fractions.

The PPH in the present study had stronger DPPH$^{\bullet}$ scavenging activity than in a previous study (*23*), in which hydrolysis of pea protein with several food grade enzymes yielded peptides with 7−11% DPPH$^{\bullet}$ scavenging activity at 1 mg/mL. The hydrolysate produced using Flavourzyme, which displayed the highest DPPH$^{\bullet}$ scavenging activity (11%), contained high concentrations of hydrophilic amino acids (asparagine, aspartic acid, glutamine, glutamic acid, arginine, and lysine) and leucine (*23*). Tang et al. (*24*) observed a range of scavenging activity of buckwheat hydrolysates from 50 to 75%, depending on the degree of hydrolysis at a concentration of 1 mg/mL. The hydrolysate with the strongest scavenging activity contained slightly higher

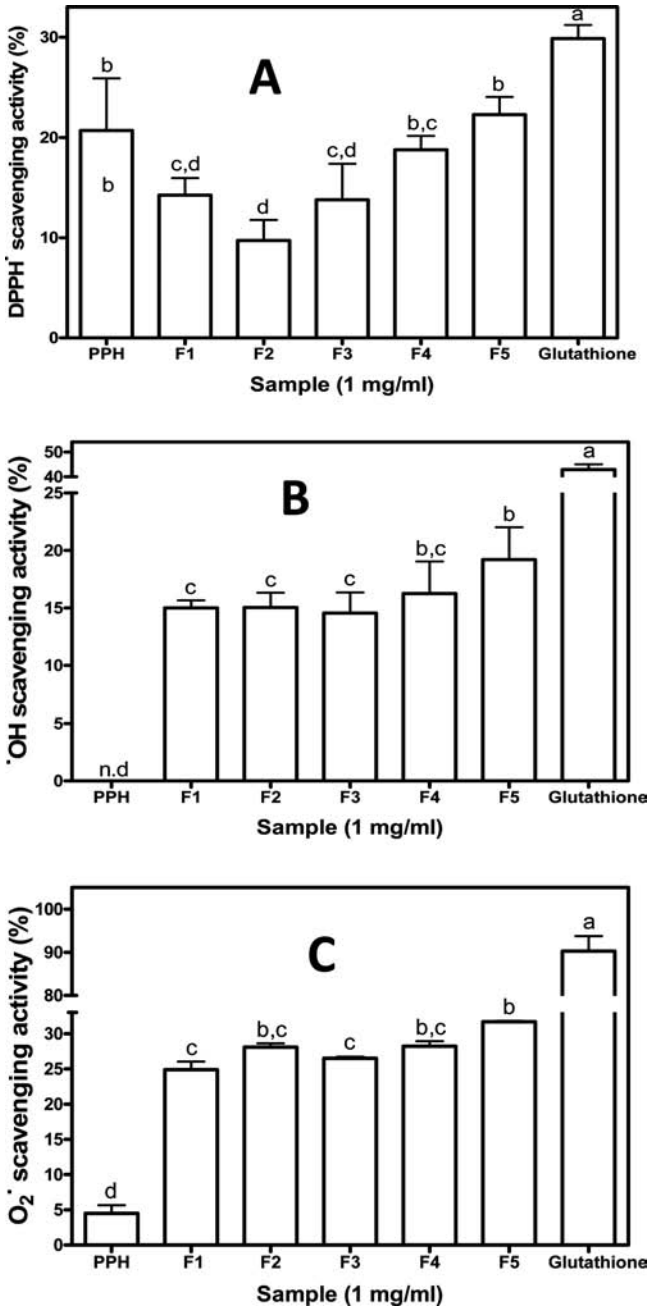

Figure 2. Free radical scavenging activities of glutathione, pea protein hydrolysate (PPH), and HPLC fractions of PPH (F1−F5): (**A**) DPPH•; (**B**) hydroxyl radical; (**C**) superoxide. Bars with different letters are significantly different at $p < 0.05$. n.d, no detected activity.

amounts of hydrophobic amino acids and had the highest surface hydrophobicity compared to hydrolysates with lower DPPH scavenging. Similarly, Li et al. (*8*) reported that a low molecular weight fraction of chickpea protein hydrolysate had strong DPPH• scavenging activity (86%) at a concentration of 1 mg/mL and also contained a high concentration of hydrophobic amino acids.

As shown in **Figure 2B**, PPH did not display OH• scavenging; however, upon fractionation by RP-HPLC, the OH• scavenging significantly increased. F5 displayed the strongest activity scavenging OH• at 17% in comparison to F1, F2, and F3 (14.5, 15, and 12.6%, respectively). The results suggest that fractionation of the PPH led to concentration of active peptides in the F4 and F5 fractions, which led to increased potency. F4 and F5 were both rich in leucine and phenylalanine and also contained higher concentrations of valine and tryptophan in comparison to F1−F3. In addition to the contribution of tryptophan to OH•-scavenging activity (*22*, *25*), phenylalanine may have also played an important antioxidant role in these peptide fractions. This is because the aromatic ring of phenylalanine can react with OH• to form stable para, meta- and ortho-substituted hydroxylated derivatives of phenylalanine (*26*). Overall, there was a strong positive correlation ($R^2 = 0.8642$) between the OH• scavenging activity and the total percent of hydrophobic amino acids of the fractions. Glutathione possessed the strongest activity (46%) for OH• scavenging. Dong et al. (*27*) observed that silver carp hydrolysates derived from Alcalase possessed stronger OH• scavenging activity and contained higher concentration of hydrophobic amino acids in comparison to a Flavourzyme hydrolysate. In particular, the concentrations of valine, methionine, isoleucine, tyrosine, phenylalanine, and proline were higher in the Alcalase hydrolysate. A low molecular weight fraction from chickpea protein hydrolysate with strong OH• scavenging activity was also observed to have higher concentrations of hydrophobic amino acids including phenylalanine, isoleucine, leucine, and valine as well as methionine and lysine in comparison to other molecular weight fractions (*8*).

As shown in **Figure 2C**, the HPLC fractions displayed moderate $O_2^{\cdot-}$ scavenging activity. F5 displayed similar $O_2^{\cdot-}$ scavenging activity as F4 and F2, whereas F1 and F3 displayed slightly lower $O_2^{\cdot-}$ scavenging activity. The PPH displayed weak superoxide scavenging activity in comparison to the fractionated peptides and glutathione. It is possible that the PPH contained several inactive peptides, which would have reduced the overall $O_2^{\cdot-}$ scavenging activity. Fractionation is a known method of concentrating peptides (*19*), and upon fractionation by RP-HPLC, it seems that the active peptides became concentrated in certain fractions, leading to stronger superoxide scavenging activity when compared to the value obtained for PPH. The pea peptide fractions F2, F4, and F5, with the highest superoxide scavenging activity, contained higher concentrations of proline in comparison to the other fractions (**Table 1**), which may have contributed to the superoxide scavenging activity of these fractions. In addition, there was a strong positive correlation between the superoxide scavenging activity of the fractions and the content of hydrophobic amino acid ($R^2 = 0.9015$).

In previous research, a low molecular weight fraction from chickpea protein hydrolysate with strong $O_2^{\cdot-}$ scavenging activity was observed to have higher concentrations of phenylalanine, isoleucine, leucine, and valine in comparison to other fractions, and it was suggested that the superoxide scavenging activity was related to the hydrophobic amino acids (*8*), a result that is similar to our present report. Xie and colleagues (*17*) demonstrated that an alfalfa leaf hydrolysate with a molecular weight of <3 kDa had strong $O_2^{\cdot-}$ scavenging activity at 67% at a concentration of 0.9 mg/mL. However, the alfalfa leaf hydrolysate contained higher concentrations of antioxidant amino acids cysteine, methionine, tyrosine, histidine, and tryptophan in comparison to the PPH examined in the present study. Overall, the peptide fractions were better scavengers of $O_2^{\cdot-}$ than hydroxyl and DPPH radicals.

H_2O_2 Scavenging. The combination of H_2O_2 with an unbound transition metal (Fe^{2+}) increases the chance of the Fenton reaction occurring and therefore leads to oxidative damage of cellular components (*28*). H_2O_2 scavenging activity of the peptide fractions displayed an increasing trend in scavenging activity with an increase in retention time (increase hydrophobic character) of the fractions as shown in **Figure 3**. F1, with the least retention time, possessed the weakest H_2O_2 scavenging activity at 40%.

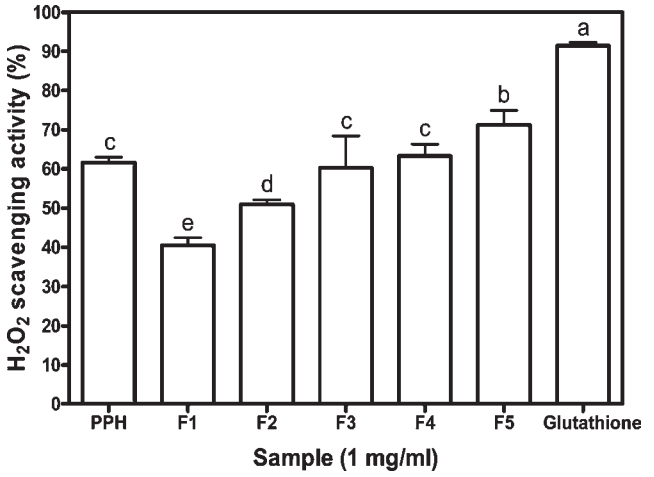

Figure 3. Hydrogen peroxide scavenging activities of glutathione, pea protein hydrolysate (PPH), and HPLC fractions of PPH (F1−F5). Bars with different letters are significantly different at $p < 0.05$.

The PPH displayed strong H_2O_2 scavenging activity; however, the activity was weaker than the scavenging activity of F5, which may have contributed most of the H_2O_2 scavenging activity of the PPH. There is a strong positive correlation between the H_2O_2 scavenging activity and the total percentage of hydrophobic amino acids of the peptide fractions ($R^2 = 0.9713$). However, reduced glutathione displayed stronger activity in comparison to the fractions.

Metal Chelation and Reducing Power. Optimal metal chelating involves aliphatic compounds, where a five-member ring is formed, which is composed of the metal ion and two chelating ligands (*29*). Histidine is considered to be a strong metal chelator due to the presence of an imidazole ring (*30*). The chelation of metal ions can decrease the amount of free iron available to participate in the Fenton reaction and ultimately decrease the formation of the $OH^{\bullet}$ (*31*). As shown in **Figure 4A**, F5 possessed the strongest metal chelating activity of the fractions. F3 and F4 displayed the same metal chelating activity at 5 and 6%, respectively, and F1, F2, and glutathione did not display metal chelating activity. Similarly, Xie et al. (*17*) observed negligible metal chelating activity of glutathione, indicating that the presence of cysteine in glutathione is not important in chelating metal ions. The PPH chelated 95% of the metal ions, indicating that it is a very effective iron chelator. The strong activity displayed by the PPH could be due to the synergistic activities of F3, F4, and F5. Fractionation of the PPH could have separated the metal chelating peptides into different fractions, thereby reducing the activity of the fractionated peptides.

Fractions F3−F5 contained higher concentrations of isoleucine, leucine, tyrosine, phenylalanine, and tryptophan (**Table 1**). F5, in particular, had the highest percentages of proline, valine, isoleucine, leucine, and phenylalanine in comparison to the other fractions. Therefore, the results suggest that the presence of aromatic rings in peptide fractions may be a contributing factor to higher metal chelation activities. The metal chelating activities of the fractions showed strong positive correlations with total aromatic amino acids ($R^2 = 0.79$) and total percentage of hydrophobic amino acids ($R^2 = 0.9498$).

In comparison, the metal chelating activity of hydrolysates derived from porcine collagen and porcine hemoglobin identified by Li et al. (*32*) and Chang et al. (*33*) ranged from 9.5 to 37% at a concentration of 11−13 mg/mL and from 50 to 64% at 5 mg/mL, respectively. Thus, the collagen and hemoglobin hydrolysates possessed weaker metal chelating activity than the PPH used in the present study. Dong et al. (*27*) observed that hydrolysates

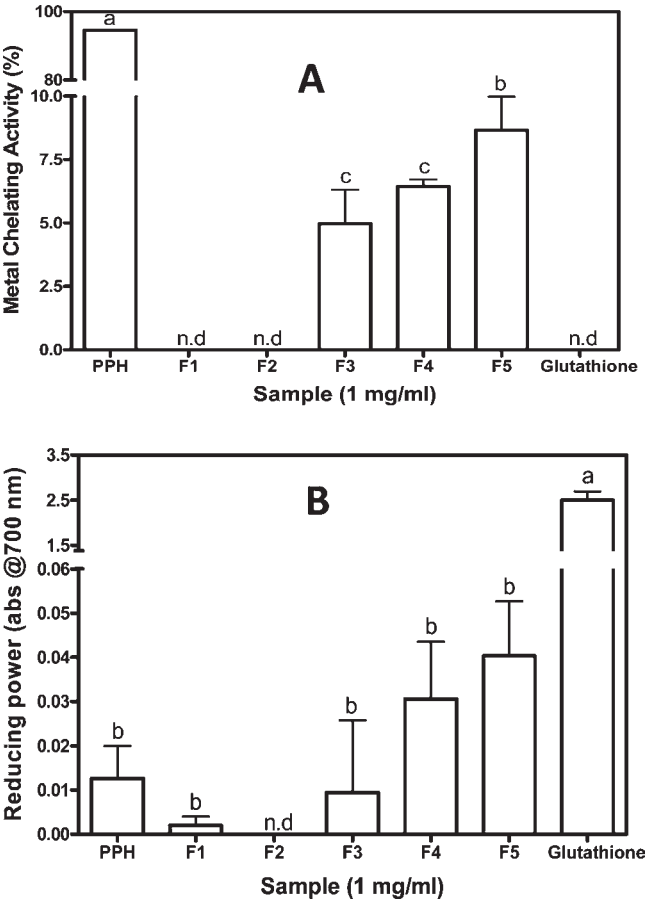

Figure 4. Metal chelating activity (**A**) and reducing power (**B**) of glutathione, pea protein hydrolysate (PPH), and HPLC fractions of PPH (F1−F5). Bars with different letters are significantly different at $p < 0.05$. n.d, no detected activity.

from silver carp, produced by different enzymes, had metal chelating activities reaching 93% at 5 mg/mL, dependent upon the type of enzyme and length of hydrolysis. The silver carp protein hydrolysate with the strongest metal chelating activity also contained higher concentrations of hydrophobic amino acids, which is similar to the present results. In contrast, Megias et al. (*30*) separated peptides from sunflower by affinity chromatography and subsequently by RP-HPLC, which produced hydrophobic fractions with no metal chelating activity. However, the least hydrophobic fractions displayed metal chelating activity and were rich in histidine (*30*). It was suggested that the imidazole ring is responsible for the strong metal chelating activity of histidine. However, in the current study, fractions with the strongest metal chelating activity had very small amounts of histidine, which is different from the results reported by Megias et al. (*30*). It is possible that the metal chelating properties of other aromatic amino acids compensated for the low level of histidine in the active pea protein hydrolysate fractions.

The ability of peptides to act as reducing agents through the donation of electrons to form more stable products was measured by the reducing power method. This method measures the ability of peptides to reduce the Fe^{3+}−ferricyanide complex to the ferrous form (Fe^{2+}) (*20*). PPH and the HPLC fractions displayed very weak reducing power when compared to glutathione as shown in **Figure 4B**. Reducing power was not detected for F2 but was positively correlated with the increase in total hydrophobic amino acids in the fractions ($R^2 = 0.9361$). In contrast, hydrolysates from smooth hound muscle displayed stronger reducing

power, which ranged from approximately 0.1 to 0.4 at a concentration of 1 mg/mL (*20*). In addition, rapeseed hydrolysate fraction separated by macroporous adsorption resin displayed good reducing power (*13*). Similar to the present results, rapeseed fractions that displayed the strongest reducing power also contained a higher amount of hydrophobic amino acids, which were suggested to be responsible for enhancing the reducing power of these peptides (*13*, *34*).

Hydrolysate derived from alfalfa leaf proteins with a molecular weight <3 kDa possessed moderate reducing power with an absorbance of 0.4 (*17*). In comparison to PPH, the <3 kDa alfalfa hydrolysate was rich in cysteine, methionine, tyrosine, histidine, and tryptophan, and it was suggested that the antioxidant activity was due to histidine, tyrosine, methionine, and cysteine contents of the peptides (*17*). PPH contained lower concentrations of these amino acids, hence its low reducing power. You et al. (*35*) observed that loach peptides hydrolyzed by papain had strong reducing power and that the hydrolysate contained tyrosine, methionine, histidine, lysine, and tryptophan. The authors also suggested that the reducing power of the peptides can be attributed to the specific amino acid and peptide composition. In the present study, glutathione displayed very strong reducing power ability, which indicates that the sulfhydryl group of cysteine is an important reducing agent. The fractions and PPH contained low amounts of sulfur-containing amino acids. On the basis of these findings, PPH and its fractions separated on the basis of hydrophobic properties do not have strong reducing power.

Inhibition of Linoleic Acid Oxidation. Previous papers have suggested that hydrophobic amino acids exhibit strong antioxidant activity to protect against lipid derived-radicals due to the ability of hydrophobic amino acids to interact with the lipids (*7*, *13*). Lipid oxidation products induce oxidation of ferrous iron to ferric iron, which reacts with ammonium thiocyanate to form a colored complex of ferric thiocyanate; therefore, absorption intensity is directly related to degree of linoleic acid oxidation (*36*). As shown in **Figure 5**, the level of linoleic acid peroxides in the control, with no added pea peptides increased rapidly and reached the highest concentration by the fourth day, which was similar to the observations of Chen et al. (*37*) and Jayaprakasha et al. (*36*). The rapid decline in absorbance of the control is believed to be due to the decomposition of (hydro)peroxides as the incubation time was increased (*36*).

PPH and the HPLC fractions exhibited strong ability to inhibit linoleic acid oxidation over 7 days (**Figure 5**). At day 1, F3 and F5 were less effective against linoleic acid oxidation as indicated by the increased absorbance. In contrast, F1, F2, F4, and PPH were slightly more effective against linoleic acid oxidation on day 1 than F3, but had the same activity as F5. By day 7, all fractions and the PPH displayed the same ability to inhibit the oxidation of linoleic acid. Glutathione initially displayed stronger activity than the pea peptide fractions. After day 2, the ability of glutathione to protect against oxidation began to decrease as indicated by the slight increase in absorption. Glutathione displayed equivalent activity to the peptide fractions and the PPH from day 3 to day 4. From day 5 to day 7, glutathione was significantly less effective ($p < 0.05$) in inhibiting linoleic acid oxidation (increased absorption values) in comparison to the peptide fractions and PPH (no change in absorption values). The decreased ability of glutathione to inhibit lipid oxidation in this test system for a prolonged period of time could be due to the fact that once glutathione has been oxidized, it forms a disulfide bridge with another glutathione. Under the test conditions, oxidized glutathione cannot be reduced and, therefore, the reduced (antioxidant form) cannot be regenerated as the experimental time is increased.

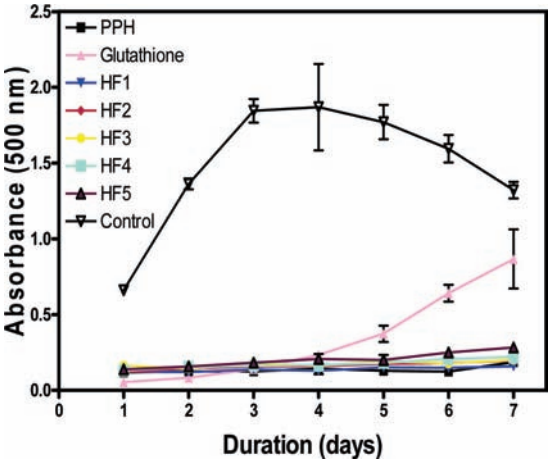

Figure 5. Inhibition of linoleic acid oxidation by glutathione, PPH, and HPLC fractions of PPH (F1−F5) at a final concentration of 1 mg/mL each.

Chen et al. (*37*) found negligible inhibition of linoleic acid peroxidation at 2 mg/mL in peanut hydrolysates, but observed activity at higher concentrations. Li et al. (*8*) identified a low molecular weight fraction from chickpea protein that contained a higher amount of hydrophobic amino acids as having the strongest activity in preventing linoleic acid oxidation. In the present work, enzymatic hydrolysis coupled with ultrafiltration and HPLC separations appears to be an effective method of producing peptide fractions with strong antioxidant activity against the oxidation of linoleic acid. Over an extended period of time, all of the peptide fractions were equally effective at inhibiting linoleic acid oxidation.

This study determined that the antioxidant activity of peptides derived from the enzymatic protein hydrolysates of yellow field pea seeds depends on the amounts of their constituent hydrophobic and aromatic amino acids. In comparison to glutathione, the pea seed peptide fractions had less ability to scavenge free radicals but better capacity to chelate metals and inhibit linoleic acid oxidation. HPLC fractionation of PPH improved reducing power and scavenging abilities against H_2O_2, superoxide, and hydroxyl radicals. In contrast, the fractionated peptides had less metal chelating but similar DPPH radical scavenging properties when compared to the PPH. The effectiveness of the PPH and fractionated peptides against linoleic acid oxidation suggests that these products could have potential roles as antioxidants against chronic diseases that are caused by high cellular oxidative stress. The results indicate that enzymatic pea seed protein hydrolysates could be used as potential ingredients to formulate functional foods and nutraceutical products.

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