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Enzymatic Hydrolysis of Oat Flour Protein Isolates to Enhance Antioxidative Properties

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Abstract: Oat is an important cereal for human consumption and has relatively higher protein content compared to other cereals. Numerous studies have shown that oat polyphenols had antioxidant properties but no data is available for similar activity on proteins and peptides. The objective of this study was to investigate the antioxidant activities of tryptic and alcalase digests of oat flour protein isolates and ultra-filtered fractions. Oat flour protein hydrolysates from alcalase (APH) and trypsin (TPH) were therefore prepared and ultrafiltered using 2 and 10 kDa molecular cutoff membranes. The free radical scavenging properties were investigated by 2,2'-diphenyl-2-picrylhydrazyl (DPPH), oxygen radical absorbance capacity, linoleic acid emulsion system and ferrous ion-chelating assays. APH and TPH significantly reduced the generation of lipid hydroperoxides resulting from autoxidation of linoleic acid after 5 days incubation. At concentration of 200 µg/L, APH and TPH also showed better chelating properties than their ultrafiltered fractions (2 kDa, 2-10 kDa). On DPPH assay recorded after 15 min alcalase fraction less than 2 kDa possessed the greater inhibition activity (32.9%) compared to 26.4% for 2 kDa trypsin fraction. The results suggest that alcalase and tryptic digests of oat flour protein can be used to produce antioxidant peptides for potential use in food products.

Key words: Antioxidant, chelating capacity, hydroperoxides, protein hydrolysates, scavenging activity, ultrafiltration

INTRODUCTION

The use of protein concentrates and isolates has attracted particular interests. As a consequence antioxidant properties of many food proteins such as bean and chickpea (Arcan and Yemenicioglu, 2007), soy (Chen *et al.*, 1998), and milk (Satué-Gracia *et al.*, 2000) are reported in the literature. It has been shown that functional and nutritional properties of these food proteins can be improved by enzymatic hydrolysis. In this respect, various physiological activities including antimicrobial, antihypertensive, immunomodulatory, opioide and mineral binding (Korhonen and Pihlanto, 2003), as well as radical scavenging activities have been found as a result proteolytic hydrolysis of food proteins. Antioxidant activities of protein hydrolysates from chickpea (Li *et al.*, 2008) and wheat germ (Zhu *et al.*, 2006) were recently reported.

Oat (*Avena sativa*) is a popular cereal for human consumption and has received increased interests because of health-related benefits associated with its intake. Bread and fruits enriched in oat beta-glucans were effective in lowering serum LDL-cholesterol level of overweight male (Reyna-Villasmil *et al.*, 2007) and healthy people (Naumann *et al.*, 2006). Phenolic compounds in oats (e.g., avenanthramides) inhibited the secretion of pro-inflammatory cytokines interleukins IL-6 and IL-8 in endothelial cells (Guo *et al.*, 2008). Oat has better protein quality compared to other cereals because of its relatively high content of lysine, a limiting amino acid in cereals (Wu *et al.*, 1977). It has been suggested that oat functional properties might not be optimum under slightly acidic conditions which are usually suitable for foods. Enzymatic hydrolysis of proteins has then been proposed as a way to improve properties like solubility, emulsification, gelation, and water holding capacity (Guan *et al.*, 2007).

Several phenolic compounds most of which are concentrated in the outer layers of oat kernel, phytic acid and vitamin E have been found to possess radical scavenging or ion-chelating activities (Peterson, 2001). There is however no report on the antioxidant potential of oat proteins. The objective of this study was therefore to find how enzymatic digestion affects antioxidant properties of oat flour proteins. We herein report on the activity of Trypsin Protein Hydrolysates (TPH), Alcalase Protein Hydrolysates (APH) and different ultrafiltered fractions. Various measurements such as the ability of the

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digests to scavenge free radicals, chelate ferrous ion and inhibit the autoxidation of linoleic acid were determined and used to evaluate antioxidant potential.

MATERIALS AND METHODS

Materials and chemicals: Oat flour was donated by Can-Oat Milling (Portage La Prairie, Manitoba MB) in September 2008 and the study was conducted in the Food Science and Nutrition laboratory at Carleton University (Canada). Linoleic acid, 2,2'-Diphenyl-1-picrylhydrazyl (DPPH), α-tocopherol, ferrozine, ethylene diamine tetraacetic (EDTA), ascorbic acid, Alcalase 2.4L (EC 3.4.21.14), trypsin (3.4.21.4), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) were purchased from Sigma-Aldrich Co. (Oakville, ON, Canada). All other chemicals used in the experiments were of analytical grade from sigma-Aldrich.

Defatted oat flour: To 60 g of oat flour 200 mL of hexanes were added and the mixture was stirred for 2 h at room temperature. The extract was filtered on Watman paper number 1 and the defatted flour dried before protein extraction.

Preparation of protein isolates: Protein isolates were prepared from defatted oat flour according to the method of Zhu *et al.* (2006) with some modifications. Defatted sample was added to a solution of 1 mol/l NaCl in a ratio of 1:8 (w/v), the pH of the slurry was adjusted to 9.5 with 1 mol/L NaOH and stirred for 30 min at room temperature. The suspension was centrifuged at 5000 *x g* for 25 min at 4°C. The supernatant was adjusted to pH 4.0 with 1.0 mol/L HCl to precipitate the proteins, and centrifuged again at 5000 *x g* for 25 min at 4ºC. The precipitates were washed (3x30 mL) with nanopure water (pH 4.0), dispersed in 15 mL of water, and adjusted to pH 7.0 with 0.1 mol/L NaOH. The sample was freeze-dried and the protein content (92%) determined using a modified Lowry assay (Markwell *et al.*, 1978).

Digestion with alcalase and trypsin: The protein isolate (300 mg) was dissolved in 20 mL of Milli-Q water at pH 8 and 220 µL of alcalase was added equivalent to an enzyme substrate ratio of 0.4 AU/g of protein. The hydrolysis was carried out at 50ºC for 4 h and stop by heating at 90ºC for 10 min.

Trypsin hydrolysis was performed similar to the method of Xu *et al.* (2007) with the following modifications. Freeze dried protein hydrolysates (300 mg) was suspended in 20 ml Milli-Q water at pH 8.0 and 5.5 mg of trypsin (enzyme/substrate ratio 1:50 w/w) were added. Incubation was carried out at 37ºC with shaking (100 rpm) for 20 h (Max Q 4000 Barnstead/Lab-Line, USA). The reaction mixture was then heated at 90ºC for 10 min to inactivate the enzyme.

The alcalase and tryptic digested samples were centrifuged at 5000 *x g* for 15 min and the supernatant ultrafiltered on 2 and 10 kDa cutoff Vivaspin™ 15R membrane from VWR Canada (Mississauga, ON). All fractions were collected and freeze dried and stored at -20ºC for further analysis.

Scavenging effect on DPPH radical: The scavenging effects of oat protein hydrolysates (APH, TPH) and ultrafiltered fractions (2 kDa, 2-10 kDa and 10 kDa) from alcalase and trypsin digests were measured according to reported procedures (Li *et al.*, 2007) with some modification. Briefly, a 60-µmol/L DPPH solution was freshly made in 100% methanol. A solution (200 µL) of each sample (0.4 and 1 mg/mL) in Milli-Q water was reacted with 0.8 ml (final sample concentration 0.08 and 0.2 mg/mL) of the DPPH solution for 15, 30, 45 and 60 min. The absorbance at 515 nm was measured using a spectrophotometer (Gary 300 UV-Visible Spectrophotometer, Varian Inc, Australia) against a blank of 100% methanol. All DPPH tests were carried out in triplicate and the antioxidant activity was calculated as follows:

$$\text{Scavenging effect (\%)} = \left(1 - \frac{A_s}{A_{CTL}}\right) \times 100$$

where, A_s was the absorbance at time t and A_{CTL} the absorbance of control (DPPH) at time zero

Oxygen radical absorbance assay of digested oat flour proteins: The assay was performed according to literature (Davalos *et al.*, 2004; Tsopmo *et al.*, 2009). An FLx800 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT) with fluorescence filters (excitation 485/20 nm, emission 528/20 nm) was used and data processed using the Gen5 software. Five Trolox concentrations between 6.25 and 100 µM were used for calibration curve. The antioxidant control was rutin trihydrate (10 µM). Protein hydrolysates and fractions (2 kDa and > 2 kDa) were analyzed at two concentrations 160 and 80 µg/mL. Data were processed as previously reported (Huang *et al.*, 2002) and the unit of measurement expressed as Trolox Equivalents (TE) per gram of sample.

Measurement of ferrous ion-chelating activity: Ferrous ion-chelating activity was determined according to the method of Dinis *et al.* (1994). Concentrations of 20, 40, 80, 120 and 200 µg/mL in Milli-Q water of protein hydrolysates and ultrafiltered fractions (2 kDa, 2-10 kDa and 10 kDa) from alcalase and trypsin digests were

prepared. For the assay, 1.5 mL of each sample was added to a freshly prepared aqueous solution of 2 mmol/L $FeCl_2$ (25 µL). The reaction was initiated by the addition of 5 mmol/L aqueous ferrozine solution (100 µL) and the mixture was shaken vigorously and left at room temperature for 10 min. The absorbance of the solution was then measured spectrophotometrically at 562 nm. EDTA was used as a positive control. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated as follows:

Ferrousion chelating activity =

$$\left(1-\frac{A_1 - A_{Blk}}{A_0}\right)\times 100$$

where, A_0 was the absorbance of the control ($FeCl_2$ and ferrozine), A_1 the absorbance in the presence of samples and A_{Blk} the absorbance of sample blank without $FeCl_2$.

Inhibition of linoleic acid autoxidation: The antioxidative activity of oat flour protein hydrolysates from alcalase (APH) and trypsin (TPH) digests with different periods of incubation was measured in a linoleic acid model system according to the methods of Osawa and Namiki (1985) with some modifications. APH and TPH (2 mg each) was dissolved in 2.5 mL of 50 mmol/L phosphate buffer (pH 7.0), and added to a solution of 30 µL linoleic acid and 2.5 mL of 99.5% ethanol for a final sample concentration of 0.4 mg/mL. The mixture was incubated in screw cap vials at 50ºC in a dark, and the degree of oxidation was evaluated by measuring the ferric thiocyanate values (Sakanaka *et al*., 2004). The reaction solution (50 µL) incubated in the linoleic acid model system described herein was mixed with 2.35 mL of 75% ethanol, 50 µL of 30% ammonium thiocyanate, and 50 µL of 0.02 mol/L ferrous chloride solution in 3.5% HCl. After 10 min, the thiocyanate value was measured by reading the absorbance (500 nm) at different intervals during 5 days incubation. α-Tocopherol was used as a control.

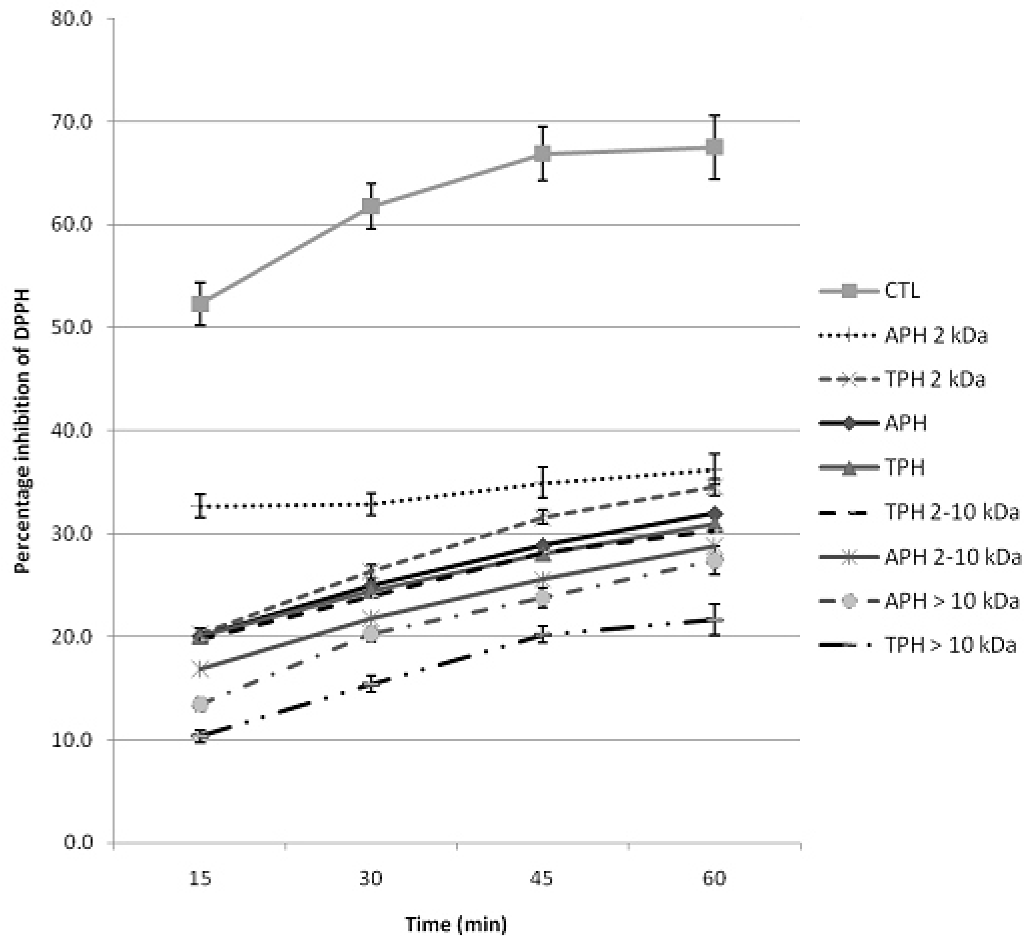

Fig. 1: Radical scavenging properties of oat flour protein hydrolyastes and ultra-filtered fractions against DPPH radicals. TPH: trypsin digest; TPH 2kDa: trypsin digest < 2 kDa; TPH 2-10kDa: trypsin digest 2 to 10 kDa; TPH: trypsin digest >10kDa

Statistical analysis: All the tests done in triplicate and absorbance were expressed as the mean±standard deviation. Differences were considered to be significant at p<0.05 as determined by one-way analysis of variance (ANOVA) using SPSSx (Version 16, 2007, Chicago Illinois).

RESULTS

Radical scavenging activity (DPPH, ORAC): DPPH is a relatively stable radical that is widely used to test the ability of compounds to scavenge free radicals and therefore act as antioxidants. We used this assay to evaluate the capacity of oat flour protein hydrolysates and ultrafiltered fractions to quench radical species (Fig. 1). Alcalase fraction 2 kDa (APH 2 kDa) was the most active and significantly quenched DPPH radicals between 15 and 45 min. APH 2kDa had 32.7 and 35% inhibition at 15 and 45 min, respectively compared to 20.3 and 31.6% inhibition for trypsin TPH 2 kDa. However at 60 min TPH 2kDa and APH 2 kDa had similar inhibition activity. The whole digests TPH and APH had similar (p>0.05) DPPH activity during the entire incubation period. Sample with the lowest scavenging activity was trypsin fraction TPH >10 kDa. The scavenging activity also determined using ORAC another commonly used assay to evaluate the antioxidant activity of food components (Davalos *et al*., 2004; Tsopmo *et al*., 2009). It was found that the ORAC values (in µmol/l trolox equivalents per gram of sample) of TPH (434±16) and APH (269±4) were significantly different. For ultrafiltered peptides fractions, the most active one was TPH >2 kDa with an ORAC value of 345±15 (Table 1).

Table 1: ORAC values of oat trypsin and alcalase digested proteins

Sample	ORAC (µM Trolox/g of sample)
TPH	434±16[a]
TPH < 2kDa	270±8[b]
TPH > 2kDa	345±15[c]
APH	269±4[b]
APH < 2kDa	149±7[d]
APH > 2kDa	241±9[e]

TPH: oat flour trypsin protein hydrolysates; TPH < 2kDa permeate form 2 kDa membrane; TPH > 2 kDa retained on 2 kDa membrane. APH oat flour alcalase protein hydrolysates; APH < 2kDa permeate form 2 kDa membrane; APH > 2 kDa retained on 2 kDa membrane. ORAC: Mean ± SD. Data with different letter indicate significant different (p<0.05)

It had been reported that amino acids tryptophan and tyrosine both containing aromatic ring moieties can be formed relatively stable radical intermediates that in turn can break radical chain reactions (Arcan and Yemenicioglu, 2007). Methionine, a sulfur-containing amino acid appeared also to be important in stopping

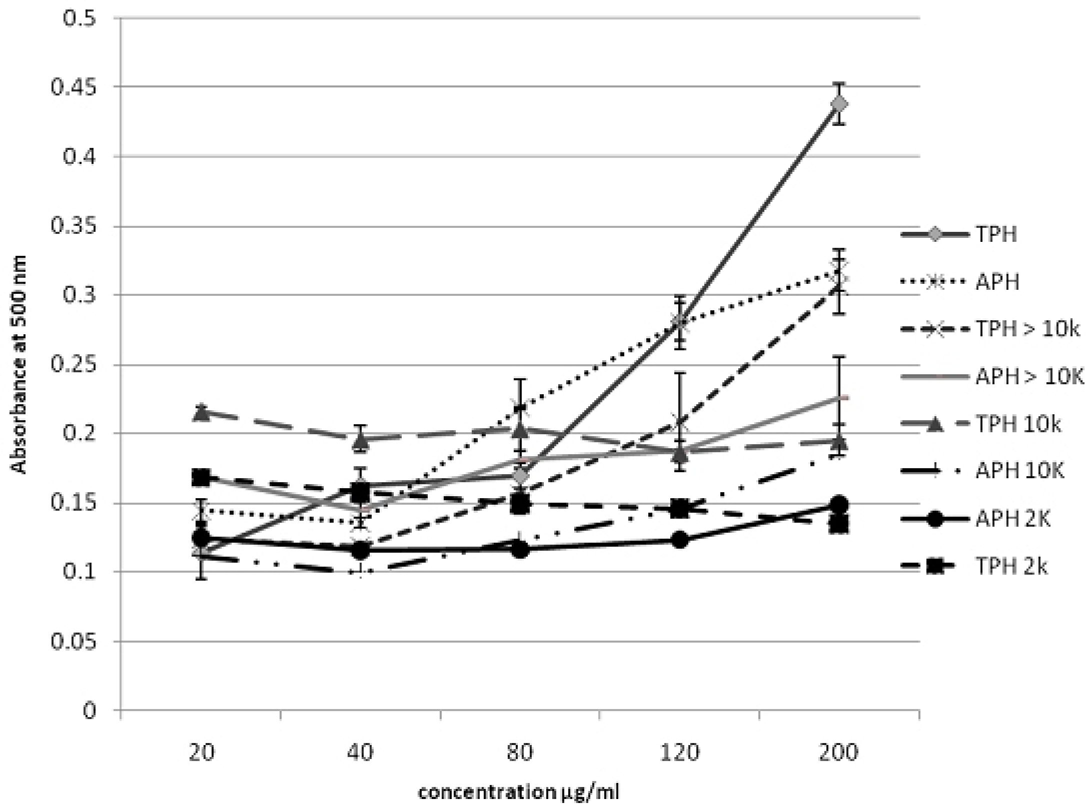

Fig. 2: Ferrous ion-chelating properties of oat flours proteins hydrolyaste and ultra-filtered fractions. TPH: trypsin digest; TPH 2kDa: trypsin digest < 2 kDa; TPH 2-10 kDa: trypsin digest 2 to 10 kDa; TPH: trypsin digest > 10 kDa. Each value is expressed as mean ± SD (*n* = 3)

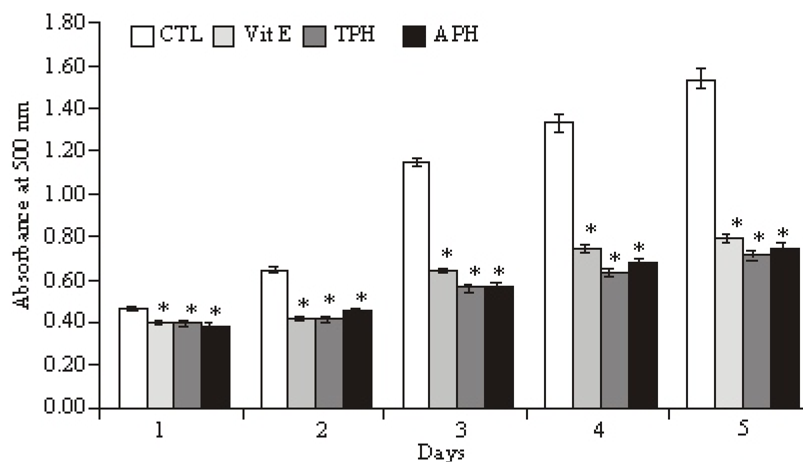

Fig. 3: Antioxidant activities of oat flour protein hydrolysates (reduction of lipid hydroperoxides) in a linoleic acid emulsion system. CTL: control, Vit E: vitamin E (α-tocopherol), TPH: trypsin protein hydrolysates, APH: alcalase protein hydrolysates. The effects of both TPH and APH were significantly *($p<0.01$) compared to control

or preventing radical reactions. The radical scavenging properties of these three amino acids are reported to be stronger compared to those of cysteine, histidine, and phenylalanine (Davalos *et al.*, 2004). The length of peptides and the position of these amino acids on the sequence may explain the difference we observed in scavenging activity for small peptide fractions (2 kDa), hydrolysates and larger peptide fractions.

Ferrous ion-chelating activity: The chelating properties of hydrolysates and peptide fractions were evaluated by monitoring the formation of the complex between ferrozine and Fe^{2+} ion spectrophotometrically at 500 nm (Fig. 2) in the presence APH, TPH and their fractions (2kDa, 2-10kDa, and >10kDa). When a compound has chelating properties, the formation of complex (Ferrozine/Fe^{2+}) is disrupted resulting in a decrease of the red colour and this makes possible the estimation of the metal chelating activity of constituents (Yamaguchi *et al.*, 2000). At 20 µg/mL the chelating effects were 16.8% for TPH 2kDa, 21.6% of TPH 2-10kDa and 11.5% for TPH. It appeared that at lower concentration, trypsin ultrafiltered fractions 2 and 2-10 kDa had better chelating properties than the whole trypsin hydrolysastes (TPH). However, increasing the sample concentration significantly increased the chelating activity of TPH (43.8% at 200 µg/L) but not the tryptic fractions. For Alcalase digests, APH >10kDa was the most active at lower concentration 16.9% (20 µg/mL) while at higher concentration it was APH with 31.8% (200 µg/mL) inhibition. Overall the trypsin digests had better chelating properties than alcalase digests of similar mass range. Certain amino acids like histidine have shown ability to chelate metals or trap lipid radicals and might contribute to peptide activities (Je *et al.*, 2004; Je *et al.*, 2005; Rajapakse *et al.*, 2005).

Linoleic acid autoxidation protection by oat protein hydrolysates: The antioxidant activities of oat flour protein hydrolysates were measured in linoleic acid emulsion system with α-tocopherol as reference. As shown in (Fig. 3), the formation of lipid peroxides from autoxidation of linoleic acid was effectively inhibited by the addition of oat flour trypsin (TPH) and alcalase (APH) protein hydrolysates at a final concentration of 0.4 mg/mL each. TPH had a slightly better inhibition than APH but this was not significantly different. The inhibition potential of both samples were close to that of α-tocopherol at the concentration of 0.4 mg/mL from day 1 to day 3 but the hydrolysates were better inhibitor of linoleic autoxidation products for days 4 and 5. Therefore, these results indicated that trypsin and alcalase hydrolysates contain antioxidative peptides that if isolated, they can be use to inhibit the formation of lipid hydroperoxides in food products.

Protein hydrolysates from other foods (Zhu *et al.*, 2006; Li *et al.*, 2008) as well as purified peptides from the enzymatic hydrolysis (Rival *et al.*, 2001; Saiga *et al.*, 2003) have been shown to inhibit the autoxidation of linoleic acid. An increase in hydrophobicity of protein hydrolysates and peptides was shown to increase peptides solubility in lipid which could enhance their antioxidative activity (Saiga *et al.*, 2003). Chen *et al.* (1998) reported that the amino acid histidine at the N-terminal had a greater contribution to the antioxidative activity of peptides. In addition, several amino acids, such as tyrosine, methionine, lysine, and tryptophan, are generally accepted as antioxidants in spite of their pro-oxidative effects in some cases (Chen *et al.*, 1996). Xiao Guan *et al.* (2007) found that the functional properties (water holding, oil holding, emulsifying, foaming) of oat barn protein concentrate

were influenced by the extent of enzymatic hydrolysis by trypsin but did not perform any antioxidant assay. It is possible that the difference in lipid peroxidation activity for TPH and APH may be due to the presence of these amino acids within the sequences.

CONCLUSION

Oat flour protein hydrolysates from trypsin (TPH) and alcalase (APH) and their respective ultra-filtered fractions (2 kDa, 2-10 kDa and >10 kDa) exhibited different ferrous ion-chelating and radical scavenging activities. The alcalase fraction APH 2kDa demonstrated the highest radical scavenging activity on DPPH assay at 15, 45 and 30 min. At 200 μg/ml trypsin digest (TPH, 43.8%) had better ferrous ion-chelating properties compared to alcalase digest (APH, 31.8%). The formation of linoleic acid autoxidation products was equally inhibited by the both trypsin and alcalase hydrolysates. From these results, it appeared that enzymatic digestion may be used to obtain radical scavenging peptides from oat flour with potential application in food products to prevent oxidative rancidity or increase nutritional values. Further research will focus on the purification of peptides from the digests for mechanistic and structure-activity relationship studies.

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