Hindawi Publishing Corporation
Journal of Chemistry
Volume 2016, Article ID 8216378, 8 pages
http://dx.doi.org/10.1155/2016/8216378

Research Article

Pepsin Digested Oat Bran Proteins: Separation, Antioxidant Activity, and Identification of New Peptides

Ariane Vanvi[1,2] and Apollinaire Tsopmo[1,3]

[1]*Food Science and Nutrition Program, Carleton University, 1125 Colonel By Drive, Ottawa, ON, Canada K1S 5B6*
[2]*National Institute of Higher Education in Agronomy, Food and Environmental Sciences, 26 Boulevard Docteur-Petitjean, BP 87999, Dijon, France*
[3]*Institute of Biochemistry, Carleton University, 1125 Colonel By Drive, Ottawa, ON, Canada K1S 5B6*

Correspondence should be addressed to Apollinaire Tsopmo; apollinaire_tsopmo@carleton.ca

Received 16 September 2015; Revised 31 December 2015; Accepted 3 January 2016

Academic Editor: Somdet Srichairatanakool

The aim of this study was to determine pepsin hydrolysis conditions to produce digested oat bran proteins with higher radical scavenging activities and separate and identify peptides. Isolated proteins were then digested with different concentrations of pepsin and incubation times. Hydrolysates produced with 1 : 30 enzyme substrate (E/S) ratio and 2 h possessed the highest peroxyl radical scavenging activity, 608 ± 17 μM TE/g (compared to 456–474 μM TE/g for other digests), and was therefore subsequently fractionated into eight fractions (F1–F8) by high performance liquid chromatography (HPLC). F1 and F2 had little activity because of their low protein contents. Activities of F3–F8 were 447–874 μM TE/g, 20–36%, and 10–14% in the peroxyl, superoxide anion, and hydroxyl radical tests, respectively. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to identify a total of fifty peptides that may have contributed to the activity of F3, a fraction that better scavenged radicals.

1. Introduction

Oxidation is a well-known phenomenon that affects the quality of food products as it creates compounds responsible for the rancid odour and changes in color or texture of some foods. Oxidised lipids can further react, for example, with proteins to form toxic compounds like pyridine, pyrrole, and other N-heterocyclic compounds [1]. In vivo, aerobic metabolism naturally produces free radicals that are eliminated by enzymes such as superoxide dismutase, catalase, and glutathione peroxidase and by molecules such as glutathione, uric acid, and bilirubin [2]. However, with aging, the decreased efficiency of the antioxidant system coupled to pollution, fatigue, or an unbalanced diet can generate free radicals in excess, thereby promoting oxidative stress [3]. The accumulation of free radicals manifests itself by causing damage to cell membrane molecules, which may then increase the risk of diseases such as cancer and cardiovascular and neurodegenerative diseases [4]. In addition to synthetic antioxidants currently available to limit oxidation, researchers are actively looking for natural antioxidants because of their ability to reduce oxidation of food and biological molecules and preference by consumers [5]. Fruits and vegetables have well been studied because of the presence of exogenous antioxidants (vitamin C, vitamin E, carotenoids, and polyphenols) that can contribute to the cellular redox balance [6]. Attention has also been focussed on the role of other food molecules and, in that respect, peptides derived from hydrolysis of food proteins are currently being investigated [7, 8]. Food peptides have then been shown to possess biological activities such as antioxidant, antihypertensive, and antimicrobial and modulation of the immune system [7, 9]. Antioxidant peptides in foods are generally released only after enzymatic hydrolysis [10] or fermentation in the presence of microorganisms [11]. Depending on their molecular weight, peptides may be transported through intestinal-expressed transporters across the enterocytes [7]. Cereals are source of fibres and polyphenols that have been extensively investigated for their effects in cardiovascular diseases and diabetes [12] but their peptides have received

less attention. Oat proteins digested with alcalase and trypsin were demonstrated to possess antioxidant activities [13, 14]. However, the action of other proteases, or the activity of fractions from chromatography separation, remains to be investigated. The objective of this study was to optimize the conditions for pepsin hydrolysis of oat bran proteins, perform HPLC separation, determine the antioxidant activity of fractions, and identify peptides.

2. Materials and Methods

2.1. Chemicals. Medium oat bran (i.d. 112-001) was supplied by Richardson Milling (Portage La Prairie, Manitoba MB). Viscozyme L® (100 Fungal Beta Glucanase (FBG)/g), pepsin from porcine gastric mucosa (367 units/mg solid), L-glutathione, Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pyrogallol, 1,10-phenanthroline, iron sulfate pentahydrate ($FeSO_4 \cdot 7H_2O$), sodium carbonate (Na_2CO_3), sodium hydroxide, sodium tartrate, cupric sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), Folin-Ciocalteu reagent, and bovine serum albumin (BSA) were obtained from Sigma Aldrich (Oakville, ON). Hydrogen peroxide (H_2O_2), 6-hydroxy-2, 5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), fluorescein, methanol, mono- and di-basic potassium phosphate, ethylenediaminetetraacetic acid, and sodium dodecyl sulfate were purchased from Fisher Scientific (Nepean, ON). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was from Wako Chemicals. Spectrophotometric and fluorometric measurements were performed on the BioTek® Epoch™ UV-Vis and Biotek FLx 800 microplate reader, respectively, both controlled by Gen5™ data analysis software. Incubations were done on a MaxQ™ 5000 shaker model (Fisher Scientific, Nepean, ON).

2.2. Protein Extraction. The extraction procedure was performed based on a previous method [14]. Samples of oat brans (4 × 100 g) were defatted by stirring in hexane (1 : 3 w/v) for 1 h at room temperature, filtration on cheesecloth, and overnight drying under fume hoods. Deionized water was added to the defatted oat bran at the ratio 1 : 10 (w/v) and adjusted to pH 4.5. Viscozyme L and 3 FBG/g of defatted bran was used to breakdown carbohydrates and reduced the viscosity. The mixture was then incubated at 45°C, 150 rpm, and 1.5 h. At the end of the incubation, the pH was adjusted to 9.5 using 2 M NaOH solution and further incubated for 1.5 h in order to carry out the alkaline extraction of proteins. After cooling, centrifugation was performed at 2500 g for 20 min at 4°C. Supernatants containing proteins were adjusted to pH 4.0 and then centrifuged (10000 g, 40 min, 4°C) to obtain protein isolates (i.e., pellets) that were suspended in pH 7.0 water before being freeze-dried.

2.3. Hydrolysis with Pepsin. Freeze-dried oat bran protein isolates (500 mg each) were transferred into twelve different tubes and rehydrated with water (1 : 12 w/v). The pH was adjusted to 4.0 with 1 M HCl followed by addition of pepsin at 1 : 20, 1 : 30, 1 : 40, and 1 : 50 (w/w) enzyme-substrate (E/S) ratios. Mixtures were homogenized, adjusted to pH 2.0, and then incubated at 37°C, 150 rpm. Four samples were removed at 1, 2, and 3 h, respectively. At the end of each incubation time, the enzyme was inactivated by adjusting the pH to 7.0 with 1 M $NaHCO_3$. Supernatants were collected after centrifugation at 2500 g for 15 min to remove denatured pepsin and nondigested proteins. The hydrolysates were freeze-dried and used for oxygen radical absorbance capacity (ORAC) assay. For HPLC separation, oat proteins (3 × 100 g) were digested with pepsin (1 : 30 E/S) for 2 h. The hydrolysate produced under these conditions had the highest ORAC value.

2.4. Oxygen Radical Absorbance Capacity (ORAC) Assay. The assay was performed using a Bio-Tek FLx800 instrument according to literature [15]. Reagents, standards, digested proteins/HPLC fractions (0.1 mg/mL), and assay control (GSH, 0.1 mg/mL) were prepared with potassium phosphate buffer (75 mM, pH 7.4). Five concentrations of Trolox (6.25–100 μM) were used to obtain the standard curve. To perform the assay, fluorescein (120 μL, 0.080 μM) was transferred into 96-well microplate followed by addition of 20 μL of samples, standards, or buffer (blank). The plate was incubated for 20 min at 37°C. AAPH (15 mM, 60 μL) was then added to each well. Data (triplicates) were collected every minute over 50 min and used to calculate ORAC values expressed as μM Trolox equivalent (TE) per gram of lyophilized hydrolysate of fraction.

2.5. Separation by RP-HPLC. Digested bran proteins (2 h, 1 : 30 E/S) with the most peroxyl radical scavenging activity were selected for HPLC separation. The system consisted of 1525 binary pump, 2998 photodiode array detector (set at 220 nm), 2707 autosampler maintained at 8°C, and fraction collector III from Waters (Montreal, QC, Canada). The freeze-dried oat bran protein hydrolysate was dissolved in 0.1% acetic acid (100 mg/mL) and filtered through 0.45 μm nylon membrane. The column was Waters Prep XBridge BEH C18, 130 Å, 10 μm, 19 × 150 mm, and the injection volume was 2 mL. A linear gradient (5 to 90% B) of 0.1% acetic acid in water (A) or in methanol (B) over 45 min was used for separation at a flow rate of 4 mL/min. Fractions (8 mL each) were collected and pooled into eight fractions according to the chromatogram: 0–6 min (F1), 6–10 min (F2), 10–18 min (F3), 18–24 min (F4), 24–28 min (F5), 28–32 min (F6), 32–36 min (F7), and 36–42 min (F8). Solvent in each fraction was removed under vacuum at 45°C using a Büchi Rotavapor® R-215, reconstituted in water, freeze-dried, and stored at −20°C. The protein content of the hydrolysate and fractions were determined using a modified Lowry method [16].

2.6. Superoxide Anion and Hydroxyl Radicals Scavenging Assays. The superoxide scavenging activity was measured according to literature [17]. Hydrolysates or peptides fractions (80 μL, 1 mg/mL) or glutathione (1 mg/mL, positive control) was mixed with 80 μL of 50 mM Tris-HCl buffer containing 1 mM EDTA (pH 8.3) into a 96-well clear microplate; then 40 μL of Pyrogallol in 10 mM HCl (1.5 mM) were added to each well. Absorbances were measured at 420 nm every 20 s

for 4 min at room temperature. The reaction rate ΔA/min was used to calculate the superoxide radical scavenging activity [18]. For determination of the hydroxyl radical scavenging activity, 50 μL of peptide fractions (1 mg/mL) in potassium phosphate buffer (0.75 mM, pH 7.4) was transferred into a 96-well clear microplate, followed by 1,10-phenanthroline in buffer (3 mM, 50 μL), $FeSO_4{\cdot}7H_2O$ in water (3 mM, 50 μL), and 0.03% aqueous H_2O_2 (50 μL). The assay control contained both phenanthroline and H_2O_2 while the blank only contained phenanthroline. The plate was incubated for 1 h at 37°C, 200 rpm. Absorbances were then read at 536 nm and used to calculate the scavenging activity [10].

2.7. Mass Spectrometry. The tandem mass spectrometry analysis was performed using the Quebec Proteomics Platform available at the Quebec Genomics Center (Sainte-Foy, QC, Canada). Peptides were identified in F3, one of the most active fractions from semiprep HPLC. Experiments were performed with an Agilent nanoscale capillary liquid chromatography (nanoLC) coupled to a triple TOF 5600 plus mass spectrometer (AB Sciex, USA) equipped with a nanoelectrospray ion source in positive mode. Peptides were separated on PicoFrit 15 μm tip, BioBasic C18, 10 cm × 75 μm column (New Objective, USA). Mobile phases were 0.1% formic acid in water (solvent A) and 0.1% acetic acid in acetonitrile (solvent B). A linear gradient 5 to 80% of solvent B in 60 min, at 300 nL/min, was used for separation. Spectra were acquired using a data-dependent acquisition mode (Analyst software version 1.6). Each full scan mass spectrum (400 to 1,250 m/z) was followed by collision-induced dissociation (MS/MS) of multiple charged peaks (2^+ to 5^+). Dynamic exclusion was set for a period of 3 s and a tolerance of 0.1 Da.

2.8. Database Searching and Criteria for Peptide Identification. Mascot generic format (MGF) files containing MS/MS peak lists were generated using Protein Pilot version 4.5 software (AB Sciex, USA). Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.4.1) and X! Tandem (The Global Proteome Machine, thegpm.org; version CYCLONE (2010.12.01.1)). Mascot was set up to search the TAX_Poeae_147387_20141216 database assuming that the digestion enzyme is nonspecific. X! Tandem was set up to search a subset of the TAX_Poeae_147387_20141216 database also assuming nonspecificity. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.100 Da and a parent ion tolerance of 0.100 Da. Dehydration of the n-terminus, glu->pyro-Glu of the n-terminus, ammonia-loss of the n-terminus, gln->pyro-Glu of the n-terminus, deamidation of asparagine and glutamine, and oxidation of methionine were specified as variable modifications in X! Tandem and Mascot to achieve a False Discovery Rate (FDR) less than 1.0% by the Scaffold Local FDR algorithm.

2.9. Statistical Analysis. All results are presented as mean ± standard deviation from replicates made in triplicate. One-way ANOVA was used and performed by SPSS (version 21, 2012). Statistical significance was set to $p < 0.05$. Means were separated using Fisher's protected least significant difference.

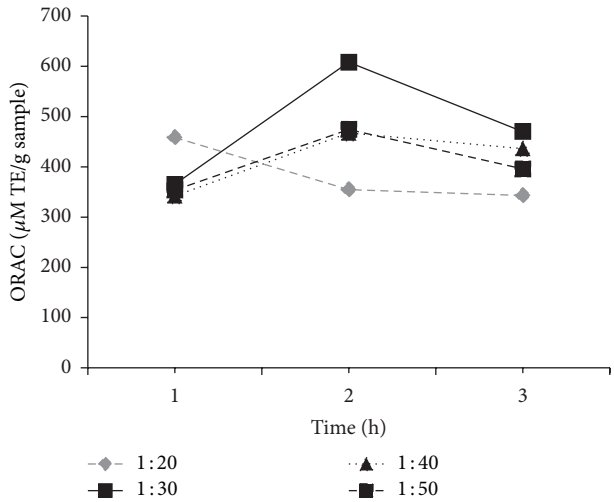

Figure 1: Effect of digestion time and enzyme substrate ratio (E/S) on the peroxyl radical scavenging activity of oat bran proteins treated with pepsin from the oxygen radical absorbance capacity (ORAC) assay.

3. Results and Discussion

3.1. Optimization of Extraction Conditions. Four concentrations of pepsin in combination with three digestion times were used to produce twelve hydrolysates from oat bran protein isolates. To determine which conditions produced samples with highest radical scavenging activity, hydrolysates were screened for peroxyl radical scavenging properties using the ORAC assay and results expressed as μM TE/g of lyophilized hydrolysate (Figure 1). After 1 h hydrolysis, proteins treated with the highest pepsin concentration (1 : 20 E/S) had higher peroxyl radical activity but the value decreased at 2 and 3 h possibly because of further degradation of the active peptides. For other E/S ratios (1 : 30–1 : 50), activities increased after 2 h digestion followed by a decrease at 3 h (Figure 1). Of all the samples, hydrolysates produced from bran proteins digested for 2 h with pepsin 1 : 30 E/S possessed the highest peroxyl radical scavenging activity (608 ± 17 μM TE/g). A large quality was then produced and subjected to semipreparative HPLC seperation. Other studies have shown that the E/S ratio and the duration of digestion had positive effect on ORAC values of hydrolyzed milk alpha-lactalbumin, caseinomacropeptide, and whey protein concentrate [19].

3.2. Separation by RP-HPLC. Enzymatic hydrolysis of food proteins is often used to produce mixtures of peptides with stronger antioxidant activities than native proteins. To further enhance the activity of digested oat bran proteins, a separation based on hydrophobicity in which molecules are eluted from the column by increasing the percentage of organic solvent was chosen because previous investigations

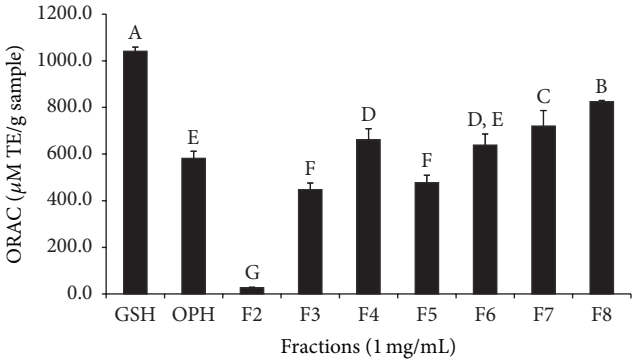

Figure 2: Oxygen radical absorbance capacity (ORAC) values of oat protein hydrolysate (OPH) hydrolyzed for 2 h with pepsin 1 : 30 E/S ratio and its RP-HPLC fractions (F2–F8). GSH: glutathione (control). Bars with different letters on the same graph are significantly different in the Fisher LSD test ($n = 3$, $p < 0.05$). Data are means ± standard deviations.

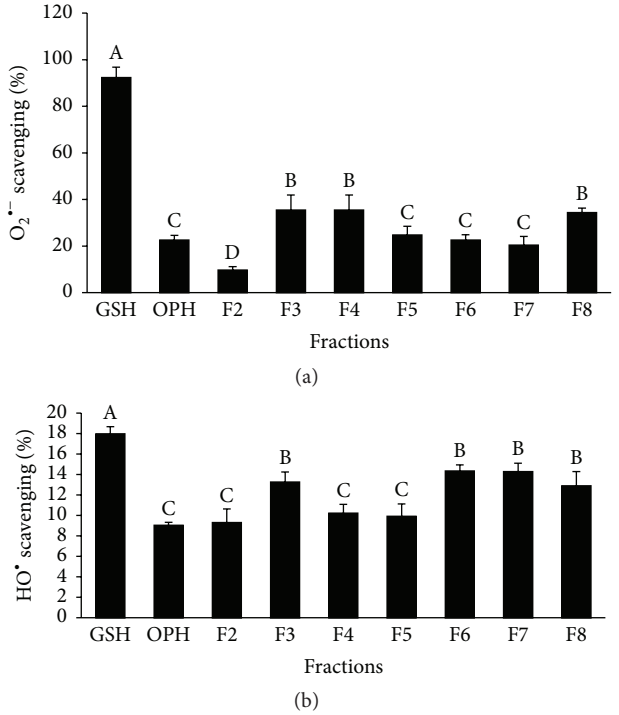

Figure 3: Superoxide (a) and hydroxyl (b) radical scavenging activities of oat protein hydrolysate (OPH) hydrolyzed for 2 h with pepsin 1 : 30 E/S ratio and its RP-HPLC fractions (F2–F8). GSH: glutathione (control). Bars with different letters on the same graph are significantly different in the Fisher LSD test ($n = 3$, $p < 0.05$). Data are means ± standard deviations.

have demonstrated the relationship between the degree of hydrophobicity and the antioxidant activity of peptides from food like soy and milk [20, 21]. A C18 column was then used to separate the pepsin hydrolyzed oat bran proteins into eight different fractions (F1–F8) as indicated in the experimental section. Less hydrophobic molecules have little interaction with the stationary phase, resulting in their rapid elution while more hydrophobic molecules bind strongly to C18 and are therefore eluted later as methanol percentage increased. Protein contents (weight/weight) of lyophilized hydrolysates determined by a modified Lowry method were 63.5 ± 0.3% nonfractionated oat protein hydrolysate (OPH), 0.0 ± 0.6% (F1), 4.0 ± 0.3% (F2), 67.9 ± 0.6% (F3), 102.2 ± 2.9% (F4), 97.6 ± 0.6% (F5), 83.5 ± 0.9% (F6), 94.6 ± 0.6% (F7), and 95.0 ± 2.5% (F8). F1 had no protein and was not further investigated. F2 had very low protein content compared to F3–F8. This is because salts used in different pH adjustments and sugars are highly water soluble and therefore will be eluted early.

3.3. Oxygen Radical Absorbance Capacity (ORAC) Assay of HPLC Fractions. The ability of antioxidant molecules to neutralize the peroxyl radical ($ROO^{\bullet}$), a common reactive species present in vivo and in food systems, is often performed using the ORAC assay [22]. The peroxyl radical quenching ability of the hydrolysate and its peptide fractions are shown in Figure 2. ORAC values significantly increased ($p < 0.005$) with elution time from F5 to F8 (477.0 ± 32.3 to 824.4 ± 5.8 μM TE/g). As organic solvent increases in the eluent, so do the degree of hydrophobicity when using a C18 column. It is therefore conceivable that there may be gradual increase of hydrophobic amino acids from F5 to F8. Although F4 had the highest protein content amongst the fractions, it did not possess the highest ORAC value, demonstrating that the peptide structure (i.e., sequence) was an important factor in scavenging radicals as suggested in other studies [7]. The presence of amino acids like tryptophan, histidine, tyrosine, or cysteine on the sequence is also important for radical scavenging properties [14] although their contents were not determined in this study. Four of the fractions possessed significantly higher scavenging power than the unfractionated oat bran protein hydrolysate (581.9 μM TE/g) indicating that hydrophobic separation enhanced their peroxyl radical scavenging activity. The highest active fraction F8 (824.4 ± 5.8 μM TE/g) was 0.8-fold the activity of control, glutathione. The activity of fractions from this study is lower than that of fractions from HPLC separation of hempseed pepsin/pancreatin protein hydrolysates [23]. Hydrolysis with both pepsin and pancreatin will result in smaller peptides compared with pepsin alone.

3.4. Superoxide Anion and Hydroxyl Radical Scavenging Activities. Superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($HO^{\bullet}$) are generally measured together because, in addition to peroxyl radicals, both are related to oxidation of foods and biological molecules. In fact, many secondary ROS are generated after reactions of $O_2^{\bullet-}$ with nonradical molecules [24, 25]. Data on the $O_2^{\bullet-}$ activity of OPH and its fractions are shown in Figure 3(a). Fractions F3, F4, and F8 possessed significantly higher ($p < 0.05$) activities (35.5 ± 6.5%, 35.5 ± 6.1%, and 34.4 ± 1.9%, resp.) than the whole hydrolysate OPH (22.6 ± 1.5%). The inhibitory activity of F5, F6, or F7 was similar to the activity of nonfractionated OPH. Fraction F2 with just 9.7 ± 1.0% protein had the least $O_2^{\bullet-}$ activity. It

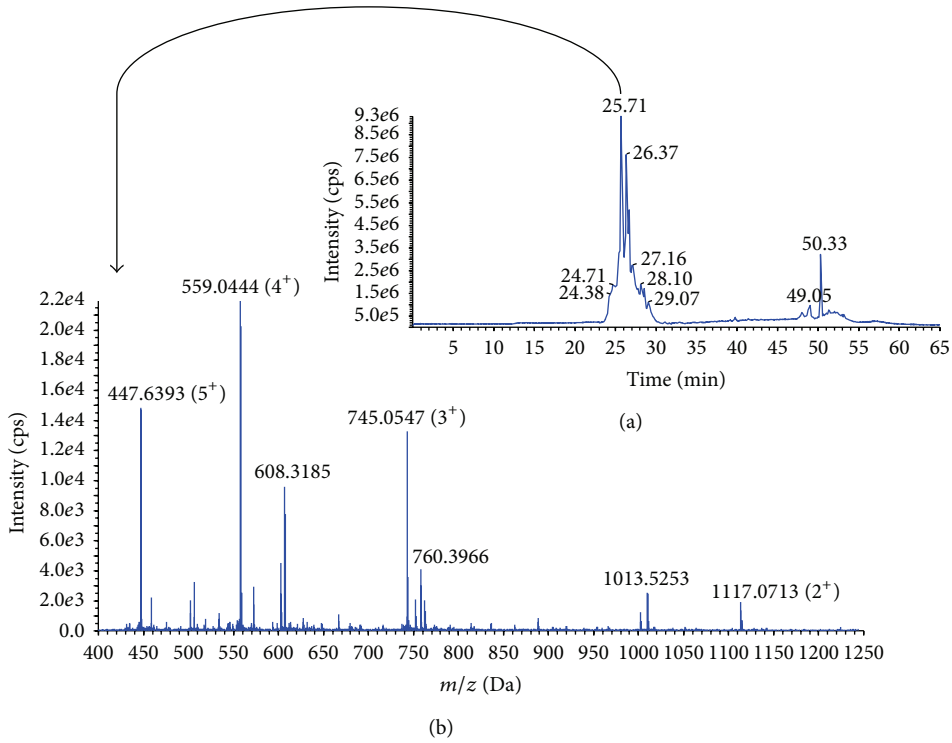

Figure 4: Mass spectrometry analysis of F3: total ions count over 65 min (a); TOF MS at 25.6 min (b). Full scan was performed from 400 to 1250 Da. Signals at *m/z* 447.63, 559.04, 745.05, and 1117.07 are quintuple, quadruple, triple, and double peaks of the same peptide (YRISRQEARNLKNNRGQE). Other peaks are for peptides with the same retention time.

appears that RP-HPLC fractionation increased the $O_2^{\bullet-}$ radical activity of some fractions compared to the unfractionated hydrolysates (OPH). None of the fraction was as active as the control GSH. In contrast with the peroxyl radical scavenging activity, there was no correction between the $O_2^{\bullet-}$ activity and the organic solvent content of the eluent suggesting that the sequence of peptides in the fractions played a more important role than hydrophobicity as reported in other studies [26, 27].

Hydroxyl radical ($HO^{\bullet}$) can be generated from $O_2^{\bullet-}$ and is considered the most damaging species in the oxidative stress process due to its high oxidizing power [27]. As displayed in Figure 3(b), fractions F3, F6, F7, and F8 have significantly higher $HO^{\bullet}$ inhibition percentages compared to OPH (9.0 ± 0.3%) but they were not as active as GSH (18.0 ± 0.7%). Fractions F2, F4, and F5 had activities comparable ($p > 0.05$) to that of OPH. Fraction F3 was obtained with an eluent high in water (60%) and mass spectrometry analysis showed that some of its peptides contained polar amino acids such as tyrosine, aspartic acid, glutamic acid, and serine (Table 1) that may have enhanced its $HO^{\bullet}$ activity through metal-chelating as reported in the literature for other peptides [28]. This is consistent with a previous study which found that later eluted HPLC (C18 column) peptide fractions were not the ones with higher $HO^{\bullet}$ scavenging activities [23].

The $O_2^{\bullet-}$ activity of fractions from this study (22.6–35.5%) is similar to that of pea peptide ultrafiltered fractions (25–32%) [17] but lower than the activity of some fractions from HPLC separation of digested salmon proteins (27–56%) [23]. The $HO^{\bullet}$ scavenging activity of the hydrolysate and its fractions are within the range (14–37%) of literature data for oat bran proteins extracted with twelve different conditions and hydrolyzed with alcalase [14]. Chickpea peptide fractions had 38 to 81% $HO^{\bullet}$ activities [10]; however the separation was performed based on size and cannot directly be compared to the present data because of differences in fractionation techniques.

3.5. Identification of Peptides. The TripleTOF® 5600 plus is a hybrid quadrupole time-of-flight hybrid instrument that offers the opportunity to conduct multiple-charge ion scanning thereby facilitating the identification of relevant functional and biological peptides in digested proteins or fractions. The system was used here to characterize peptides in one of the fractions (i.e., F3) with higher $O_2^{\bullet-}$ and $HO^{\bullet}$ radical scavenging activities. Multiple charge (2^+ to 5^+) scanning was performed over 65 min on eluates from the nanoLC system. MS/MS peak lists were analyzed using Mascot™ and X! Tandem as described in Section 2. Search results from Mascot and X! Tandem were statistically interpreted by Scaffold, a software used to validate MS/MS data. The software peptide prophet algorithm converts search engine scores into combined probabilities that improve the sensitivity and the accuracy of the identification [29]. In the total ion count chromatogram of F3 (Figure 4(a)), peptide peaks appeared between 23 and 31 min. The MS scan at 25.7 min (Figure 4(b)) showed double, triple, quadruple, and quintuple ions of the same peptide at *m/z* 1117.07 (2^+), 745.05

Table 1: List of peptides identified in F3, a fraction with higher superoxide anion and hydroxyl radical scavenging activities, from HPLC separation of pepsin digested oat brans proteins on a C18 column. Scaffold software was used to validate MS/MS data. Peptide identifications were accepted if they could be established at greater than 95% probability.

Peptide sequence	Observed mass (charge)	Actual mass	Oat protein ID, UniProt database
RALPVDVL	441.77 (+2)	881.53	P12615, P14812, P27919
SPYWNINA	482.73 (+2)	963.44	Q38781, P12615, P14812
PQYHNAPGLV	548.28 (+2)	1,094.55	O49257, O49258, P12615, P14812
HGQNFPILNL	576.81 (+2)	1,151.61	O49257, O49258
NSKNFPILNI	580.33 (+2)	1,158.64	Q38779, Q38780
VYILQGRGFTG	605.83 (+2)	1,209.65	Q38779, Q38780, P12615, P14812
VYLLQGRGFTG	605.83 (+2)	1,209.65	O49257
VIRRVIEPQGLL	464.96 (+3)	1,391.86	O49257, O49258, P12615, P14812
IRRVIEPQGLLL	469.63 (+3)	1,405.88	O49257, O49258, P12615, P14812
QQVFQPQQQAQF	738.86 (+2)	1,475.71	I4EP64, I4EP88, F2Q9W3, F4MJY2
SVIRRVIEPQGLL	493.97 (+3)	1,478.90	O49257, P12615, P14812
PAGIVHWGYNDGDAPVVA	919.44 (+2)	1,836.87	O49257, O49258
QAAQRIQSQKEQRGEI	618.33 (+3)	1,851.96	O49257, O49258
KTNPNSMVSHIAGKSSIL	628.67 (+3)	1,882.99	O49257, O49258, Q38780, P27919
IQGHARVQVVNNNGQTVF	661.02 (+3)	1,980.03	O49257, Q38779, Q38780
IQGRARVQVVNNHGQTVF	506.53 (+4)	2,022.09	Q38781, P12615, P14812
DVNNNANQLEPRQKEFL	677.01 (+3)	2,028.00	O49257, O49258, P12615, P14812, Q38780
AEGQSQSQNLKDEHQRVH	523.50 (+4)	2,089.99	P14812
YRISRQESQNLKNNRGEE	556.03 (+4)	2,220.10	P12615, Q38781
YRISRQEARNLKNNRGEE	1117.08 (+2)	2,232.14	Q38779, Q38780
YRISRQEARNLKNNRGQE	559.04 (+4)	2,232.15	O49258, P27919
YRISRQEARNLKNNRGQES	580.56 (+4)	2,318.19	O49258, P27919
YRISRQEARNLKNNRGQESG	594.81 (+4)	2,375.22	O49258, P27919
NAYRISRQESQNLKNNRGEE	802.73 (+3)	2,405.18	P12615, Q38781
NAYRISRQEARNLKNNRGQE	605.31 (+4)	2,417.23	O49258, P27919
NAYRISRQEARNLKNNRGEE	605.31 (+4)	2,417.23	Q38779, Q38780
YRISRQEARNLKNNRGQESGV	619.58 (+4)	2,474.28	O49258, P27919
ANAYRISRQEARNLKNNRGQE	623.07 (+4)	2,488.26	O49258, P27919
ANAYRISRQEARNLKNNRGEE	623.07 (+4)	2,488.26	Q38779, Q38780
NAYRISRQEARNLKNNRGQES	626.83 (+4)	2,503.27	O49258, P27919
NAYRISRQEARNLKNNRGQESG	854.44 (+3)	2,560.30	O49258, P27919
ANAYRISRQEARNLKNNRGQES	644.59 (+4)	2,574.31	O49258, P27919
RQNIENPKRADTYNPRAGRITH	652.60 (+4)	2,606.35	Q38781, P14812, P12615, O49257
ANAYRISRQEARNLKNNRGQESG	658.84 (+4)	2,631.33	O49258, P27919
ARQNIENPKRADTYNPRAGRITH	536.49 (+5)	2,677.39	Q38781, P14812, P12615
RALPIDVL	448.78 (+2)	895.55	Q38779, Q38780
IRRVIEPQGLL	431.94 (+3)	1,292.79	O49257_AVESA
QQQFQPFDQAQ	682.82 (+2)	1,363.62	P14812, O49258
LIVPQHY	435.25 (+2)	868.48	O49258
LLLPQYH	442.26 (+2)	882.50	O49257, O49258, P14812, P12615
LIVPQHF	427.25 (+2)	852.49	Q38779, Q38780
HGQNFPIL	463.25 (+2)	924.48	O49257_AVESA, O49258
SPFWNINA	474.73 (+2)	947.45	O49257, O49258, Q38780, Q38779

Q38779: 11S globulin, Q38780: 11S globulin, Q38781: oat storage 12S globulin, O49257: 12S globulin, O49258: 12S globulin, P12615: 12S seed storage globulin 1, P14812: 12S seed storage globulin 2, P27919: avenin, I4EP88: avenin, I4EP64: avenin, F2Q9W3: avenin protein, and F4MJY2: avenin protein.

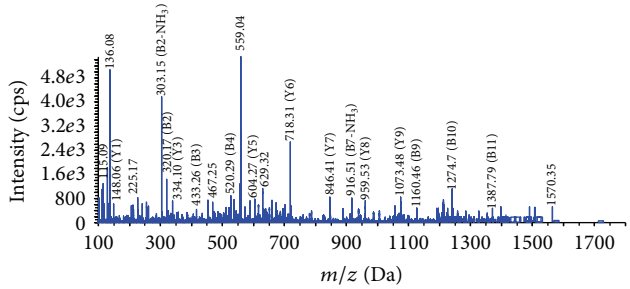

Figure 5: TOF product of the peak at m/z 559.04. The sequence was elucidated as YRISRQEARNLKNNRGQE. The main peaks B- and Y-ions are indicated in parenthesis. TOF products of peaks at m/z 1117.08 (2^+) and 447.43 (5^+), 745 (3^+) afforded the same sequence.

(3^+), 559.04 (4^+), and 447.63 (5^+), respectively. Products spectra (MS/MS) of each of these ions and database searches all correspond to the sequence YRISRQEARNLKNNRGQE (Tyr-Arg-Ile-Ser-Arg-Gln-Glu-Ala-Arg-Asn-Leu-Lys-Asn-Asn-Arg-Gly-Gln-Glu) with 100% Scaffold probability. The product ion spectrum (MS/MS) of the quadruple charged peak at m/z 559.04 can be seen in Figure 5. The list of all identified peptides in this study is shown in Table 1. They are derived from 12S globulin, 11S globulin, 12S seed storage globulin, avenin, and gliadin like avenin proteins. Most of the peptides are from 12S globulin with minor variation in sequences. Amino acids like tyrosine, cysteine, methionine, and arginine present in many identified peptides may have contributed to the activity of fraction F3 as they are known to contribute to the antioxidant activity of peptides [30]. The contribution of these amino acids is however not known at this time because they were not individually tested and also because MS/MS data are only quantitative. Peptides reported here are different from Tyr-His-Asn-Ala-Pro-Gly-Leu-Val-Tyr-Ile-Leu, Asp-Val-Asn-Asn-Asn-Ala-Asn-Gln-Leu-Glu-Pro-Arg, Gly-Gln-Thr-Val-Phe-Asn-Asp-Arg-Leu-Arg-Gln-Gly-Gln-Leu-Leu, and Val-Val-Asn-Asn-Asn-Gly-Gln-Thr-Val-Phe-Asn-Asp-Arg-Leu-Arg-Gln-Gly-Gln-Leu-Leu recently identified in oats [14]. Two of the peptides Ser-Pro-Phe-Trp-Asn-Ile-Asn-Ala (SPFWNINA) and Gln-Gln-Pro-Ile-Pro-Gln-Gln-Pro-Gln (QQPIPQQPQ) have been predicted as potential antihypertensive and celiac toxic from barley hordein and wheat omega-gliadin, respectively [31]. Other peptides present in the literature from oats are mainly made of two or three amino acids [32].

4. Conclusion

Results from this study showed that optimum conditions to produce digested proteins with the highest peroxyl radical activity were treatment with pepsin 1 : 30 E/S ration followed by 2 h incubation. RP-HPLC fractionation of hydrolysed proteins under the above conditions enhanced peroxyl, superoxide anion, and hydroxyl radical scavenging activities of some fractions. New peptides were identified by tandem mass spectrometry. In future studies, chemometric methods will be used to identify those peptides with potential bioactive activity for synthesis and evaluation of their capacity to prevent oxidation in vitro and in cell culture models.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

This work was supported by a grant from National Science and Engineering Research Council of Canada (Grant no. 371908). The authors thank Mr. Daniel Defoy from the Quebec Proteomics Platform available at the Quebec Genomics Center for technical assistance.

References

[1] S. Kubow, "Routes of formation and toxic consequences of lipid oxidation products in foods," *Free Radical Biology & Medicine*, vol. 12, no. 1, pp. 63–81, 1992.

[2] A. M. Rizzo, P. Berselli, S. Zava et al., "Endogenous antioxidants and radical scavengers," *Advances in Experimental Medicine and Biology*, vol. 698, pp. 52–67, 2010.

[3] A. G. P. Samaranayaka and E. C. Y. Li-Chan, "Food-derived peptidic antioxidants: a review of their production, assessment, and potential applications," *Journal of Functional Foods*, vol. 3, no. 4, pp. 229–254, 2011.

[4] M. Valko, D. Leibfritz, J. Moncol, M. T. D. Cronin, M. Mazur, and J. Telser, "Free radicals and antioxidants in normal physiological functions and human disease," *International Journal of Biochemistry & Cell Biology*, vol. 39, no. 1, pp. 44–84, 2007.

[5] D. Dziki, R. Rózyło, U. Gawlik-Dziki, and M. Świeca, "Current trends in the enhancement of antioxidant activity of wheat bread by the addition of plant materials rich in phenolic compounds," *Trends in Food Science and Technology*, vol. 40, no. 1, pp. 48–61, 2014.

[6] D. M. Peterson, "Oat antioxidants," *Journal of Cereal Science*, vol. 33, no. 2, pp. 115–129, 2001.

[7] C. C. Udenigwe and R. E. Aluko, "Food protein-derived bioactive peptides: production, processing, and potential health benefits," *Journal of Food Science*, vol. 77, no. 1, pp. R11–R24, 2012.

[8] S. K. Kim, I. Wijesekara, E. Y. Park, Y. Matsumura, Y. Nakamura, and S. Kenji, "Bioactive proteins and peptides," in *Bioactive Food Proteins and Peptides: Applications in Human Health*, N. S. Hettiarachchy, S. Sato, M. R. Marshall, and A. Kannan, Eds., pp. 97–116, CRC Press, Boca Raton, Fla, USA, 2011.

[9] L. Zhu, C. Jie, X. Tang, and Y. L. Xiong, "Reducing, radical scavenging, and chelation properties of in vitro digests of alcalase-treated zein hydrolysate," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 8, pp. 2714–2721, 2008.

[10] Y. H. Li, B. Jiang, T. Zhang, W. M. Mu, and J. Liu, "Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate (CPH)," *Food Chemistry*, vol. 106, no. 2, pp. 444–450, 2008.

[11] M. C. García, P. Puchalska, C. Esteve, and M. L. Marina, "Vegetable foods: a cheap source of proteins and peptides with antihypertensive, antioxidant, and other less occurrence bioactivities," *Talanta*, vol. 106, pp. 328–349, 2013.

[12] T. Madhujith and F. Shahidi, "Antioxidative and antiproliferative properties of selected barley (*Hordeum vulgarae* L.) cultivars and their potential for inhibition of Low-Density Lipoprotein (LDL) cholesterol oxidation," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 13, pp. 5018–5024, 2007.

[13] A. Tsopmo, A. Cooper, and S. Jodayree, "Enzymatic hydrolysis of oat flour protein isolates to enhance antioxidative properties," *Advance Journal of Food Science and Technology*, vol. 4, no. 4, pp. 206–212, 2010.

[14] S. Jodayree, J. C. Smith, and A. Tsopmo, "Use of carbohydrase to enhance protein extraction efficiency and antioxidative properties of oat bran protein hydrolysates," *Food Research International*, vol. 46, no. 1, pp. 69–75, 2012.

[15] D. Huang, B. Ou, M. Hampsch-Woodill, J. A. Flanagan, and E. K. Deemer, "Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated β-cyclodextrin as the solubility enhancer," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 7, pp. 1815–1821, 2002.

[16] M. A. K. Markwell, S. M. Haas, L. L. Bieber, and N. E. Tolbert, "A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples," *Analytical Biochemistry*, vol. 87, no. 1, pp. 206–210, 1978.

[17] T. L. Pownall, C. C. Udenigwe, and R. E. Aluko, "Amino acid composition and antioxidant properties of pea seed (*Pisum sativum* L.) Enzymatic protein hydrolysate fractions," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 8, pp. 4712–4718, 2010.

[18] R. Alrahmany and A. Tsopmo, "Role of carbohydrases on the release of reducing sugar, total phenolics and on antioxidant properties of oat bran," *Food Chemistry*, vol. 132, no. 1, pp. 413–418, 2012.

[19] T. G. Tavares, M. M. Contreras, M. Amorim et al., "Optimisation, by response surface methodology, of degree of hydrolysis and antioxidant and ACE-inhibitory activities of whey protein hydrolysates obtained with cardoon extract," *International Dairy Journal*, vol. 21, no. 12, pp. 926–933, 2011.

[20] H.-M. Chen, K. Muramoto, and F. Yamauchi, "Structural analysis of antioxidative peptides from soybean β-conglycinin," *Journal of Agricultural and Food Chemistry*, vol. 43, no. 3, pp. 574–578, 1995.

[21] B. Hernández-Ledesma, A. Dávalos, B. Bartolomé, and L. Amigo, "Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-MS/MS," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 3, pp. 588–593, 2005.

[22] R. L. Prior, X. Wu, and K. Schaich, "Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 10, pp. 4290–4302, 2005.

[23] A. T. Girgih, C. C. Udenigwe, and R. E. Aluko, "Reverse-phase HPLC separation of hemp seed (*Cannabis sativa* L.) protein hydrolysate produced peptide fractions with enhanced antioxidant capacity," *Plant Foods for Human Nutrition*, vol. 68, no. 1, pp. 39–46, 2013.

[24] L. Magnani, E. M. Gaydou, and J. C. Hubaud, "Spectrophotometric measurement of antioxidant properties of flavones and flavonols against superoxide anion," *Analytica Chimica Acta*, vol. 411, no. 1-2, pp. 209–216, 2000.

[25] C. F. Ajibola, J. B. Fashakin, T. N. Fagbemi, and R. E. Aluko, "Effect of peptide size on antioxidant properties of African yam bean seed (*Sphenostylis stenocarpa*) protein hydrolysate fractions," *International Journal of Molecular Sciences*, vol. 12, no. 10, pp. 6685–6702, 2011.

[26] X. Li, "Improved pyrogallol autoxidation method: a reliable and cheap superoxide-scavenging assay suitable for all antioxidants," *Journal of Agricultural and Food Chemistry*, vol. 60, no. 25, pp. 6418–6424, 2012.

[27] M. Laguerre, J. Lecomte, and P. Villeneuve, "Evaluation of the ability of antioxidants to counteract lipid oxidation: existing methods, new trends and challenges," *Progress in Lipid Research*, vol. 46, no. 5, pp. 244–282, 2007.

[28] K. M. Chan and E. A. Decker, "Endogenous skeletal muscle antioxidants," *Critical Reviews in Food Science and Nutrition*, vol. 34, no. 4, pp. 403–426, 1994.

[29] B. C. Searle, "Scaffold: a bioinformatic tool for validating MS/MS-based proteomic studies," *Proteomics*, vol. 10, no. 6, pp. 1265–1269, 2010.

[30] A. McDermott, *Bioactive Peptides*, Springer Science & Business Media, New York, NY, USA, 2009.

[31] A. Cavazos and E. Gonzalez de Mejia, "Identification of bioactive peptides from cereal storage proteins and their potential role in prevention of chronic diseases," *Comprehensive Reviews in Food Science and Food Safety*, vol. 12, no. 4, pp. 364–380, 2013.

[32] I. W. Y. Cheung, S. Nakayama, M. N. K. Hsu, A. G. P. Samaranayaka, and E. C. Y. Li-Chan, "Angiotensin-I converting enzyme inhibitory activity of hydrolysates from oat (*Avena sativa*) proteins by in silico and in vitro analyses," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 19, pp. 9234–9242, 2009.