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Flaxseed protein-derived peptide fractions: Antioxidant properties and inhibition of lipopolysaccharide-induced nitric oxide production in murine macrophages

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ABSTRACT

A protein isolate was produced from cellulase-treated defatted flaxseed meal followed by hydrolysis with seven proteases and evaluation of the hydrolysates for antioxidant and anti-inflammatory properties. The flaxseed protein hydrolysates (FPH) were processed by ultrafiltration and ion-exchange chromatography to isolate low molecular weight (LMW) and cationic peptide fractions, respectively. The peptides showed antioxidant properties in scavenging 2,2-diphenyl-1-picrylhydrazyl radical, superoxide anion radical, electron-spin resonance-detected hydroxyl radical and nitric oxide. In addition, all peptide fractions inhibited semicarbazide-sensitive amine oxidase activity. Antioxidant activities of these peptides were dependent on the specificity of proteases and size of the resulting peptides. The LMW fractions from pepsin, ficin and papain FPH also inhibited lipopolysaccharide-induced nitric oxide productions in RAW 264.7 macrophages without apparent cytotoxicity; thus, these peptides may act as anti-inflammatory agents. Thus, flaxseed protein hydrolysates may serve as potential ingredients for the formulation of therapeutic products.

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1. Introduction

Reactive oxygen species (ROS) from dietary sources, cellular processes and mitogen-activated immune cells have been shown to be associated with the aetiology and pathogenesis of human physiological and disease conditions due to their roles in the oxidative degradation of biological macromolecules (Ames, 1983; Ames, Shigena, & Hegen, 1993; Pacher, Beckman, & Liaudet, 2007). Epidemiological studies have shown a positive correlation between consumption of fruits and vegetables, which are rich sources of antioxidants, and reduction of risk of some ROS-mediated diseases (Kris-Etherton et al., 2002). The predominant ROS and reactive nitrogen intermediates generated from human physiological processes include hydrogen peroxide (H_2O_2), superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), nitric oxide (NO) and peroxynitrite ($ONOO^-$). NO is a signaling molecule responsible for the regulation of physiological processes including vasodilation and neurotransmission (Pacher et al., 2007). It is synthesised in various cell types from L-arginine and molecular oxygen by a group of enzymes known as nitric oxide synthases (NOS). In macrophages and neutrophils, NO is produced in large amounts by calcium/calmodulin (CaM)-independent inducible NOS (iNOS) in response to pro-inflammatory cytokines and endotoxins. In the cells, NO may react with $O_2^{\cdot-}$ to form a more reactive DNA-damaging $ONOO^-$, which could also be converted to $^{\cdot}OH$ (Pacher et al., 2007). These highly reactive intermediates could trigger degenerative cellular processes including inflammation-related tissue damage such as arthritis (Ames, 1983; Ames et al., 1993; Liu et al., 2002; Pacher et al., 2007; Torre, Pugliese, & Speranza, 2002). In addition, overproduction of NO in macrophages has also been shown to play a crucial role in the pathogenesis of human immunodeficiency virus (HIV)-1 infection (Torre et al., 2002). Therefore, suppression of mitogen-induced cellular nitric oxide production could constitute a step in treatment of inflammatory diseases (Liu et al., 2002) and in suppressing virus-induced pathogenesis in HIV-1 infection (Torre et al., 2002).

Semicarbazide-sensitive amine oxidase (SSAO, E.C.1.4.3.6.) is the name of a group of enzymes ubiquitously distributed in plants and animals. Amongst its several physiological roles, it catalyses the oxidative deamination of various primary amines to produce their respective aldehydes, ammonia and H_2O_2 (Göktürk et al., 2003; Magyar, Mészáros, & Mátyus, 2001; Stolen, Madanat, et al., 2004; Stolen, Yegutkin, et al., 2004). Methylamine and aminoacetone are well-known endogenous substrates for SSAO; their deamination products are potential cytotoxic agents and precursors of

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advanced glycation end-products (AGEs) (Göktürk et al., 2003; Stolen, Madanat, et al., 2004; Stolen, Yegutkin, et al., 2004). Plasma level of SSAO has been reported to be elevated in disease conditions such as diabetes mellitus, atherosclerosis, cerebral infarction, liver cirrhosis and congestive heart failure (Boomsma, De Kam, Tjeerdsma, Van Den Meiracker, & Van Veldhuisen, 2000; Göktürk et al., 2003; Magyar et al., 2001; Stolen, Madanat, et al., 2004; Stolen, Yegutkin et al., 2004). The increased serum activity of SSAO has been associated with progression of vascular endothelial damages mediated by AGEs (Stolen, Yegutkin, et al., 2004). Based on these findings, it was proposed that inhibition of SSAO could reduce the damages observed in these disease conditions (Göktürk et al., 2003; Magyar et al., 2001); moreover, SSAO inhibitory activities have been reported for natural and synthetic compounds and biomaterials (Lin, Wang, Lu, Wu, & Hou, 2008; Liu, Wu, Liang, & Hou, 2007).

Flaxseed (*Linum usitatissimum*) is an oilseed found in various parts of the world including Canada, producer of the largest amount of the world export market of flaxseed (Agriculture and Agri-Food Canada, 2007). It has been widely studied for its abundant α-linolenic acid, dietary fibre and lignan constituents, which have been reported to possess potential to reduce the risk of cardiovascular disease in human (Dodin et al., 2008). However, the protein components of flaxseed have not been optimally utilised especially in human nutrition. Flaxseed proteins contain high amounts of arginine, lysine and branch-chain amino acids (Hall, Tulbek, & Xu, 2006; Oomah & Mazza, 1993). Due to their amino acid profiles, there are considerable interests in value-added use of flaxseed proteins isolated from defatted flaxseed meal. Thus, previous studies have reported that cationic peptides from Alcalase-catalysed flaxseed protein hydrolysate (FPH) bound and inactivated CaM with concomitant inhibition of endothelial and neuronal NOS (Omoni & Aluko,2006a, 2006b). Moreover, it has also been reported that Flavourzyme-catalysed FPH inhibited angiotensin-converting enzyme activity, and showed antioxidant properties in scavenging ($O_2^{\cdot-}$) and lipid peroxyl radical (Marambe, Shand, & Wanasundara, 2008). The aim of this study was to determine the antioxidant and anti-inflammatory properties of low molecular weight (LMW) and cationic peptide fractions from flaxseed proteins that have been hydrolysed with various food-grade and human gastrointestinal tract proteases. The inhibitory activity of the peptides against lipopolysaccharide (LPS)-induced nitric oxide production in RAW 264.7 macrophages was used as a model to determine the potential anti-inflammatory properties of these flaxseed peptide fractions.

2. Materials and methods

2.1. Materials

Defatted flaxseed meal was a gift from Bioriginal Foods and Science Corporation (Saskatoon, SK, Canada). Cellulase (*Aspergillus niger*), Alcalase (*Bacillus licheniformis*), thermolysin (*Bacillus thermoproteolyticus rokko*), ficin (fig tree latex), pepsin (porcine gastric mucosa), trypsin (bovine pancreas), papain (papaya latex), pancreatin (porcine pancreas), pyrogallol (1,2,3-trihydroxybenzene), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS), benzylamine, bovine plasma, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferrous sulphate, horseradish peroxidase (148 units/mg solid), lipopolysaccharide (LPS), sodium nitroprusside (SNP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT), polymyxin B (PMB) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Hydrogen peroxide (33%) was from Wako Pure Chemical Industry (Osaka, Japan). Other analytical grade reagents were obtained from Fischer Scientific (Oakville, ON, Canada).

2.2. Production of FPH and cationic peptide fractions

The protein components of the defatted flaxseed meal were isolated using a new method as previously reported (Udenigwe, Lin, Hou, & Aluko, 2009). Briefly, defatted flaxseed meal (5%, w/v, dry weight basis) was suspended in deionized water and stirred thoroughly using a magnetic stirrer. The highly viscous slurry was adjusted to pH 5.0 and a temperature of 37 °C followed by addition of cellulase (1%, w/w; activity of powder, 1.44 U/mg) to initiate fibre hydrolysis. After 4 h of reaction, the resulting suspension was cooled to 4 °C followed by alkaline solubilisation (pH 10.0) with 0.5 M NaOH and acid-induced protein precipitation at pH 4.2 with 0.5 M HCl following the method of Dev and Quensel (1988). The resulting protein precipitate was washed thrice with acidified water (pH 4.2), suspended in a small volume of deionized water and the pH adjusted to 7.0 using 0.5 M NaOH. The suspension was freeze-dried and stored at −20 °C.

Hydrolysis of the protein fractions of flaxseed meal was conducted under different conditions using a pH-stat instrument (Metrohm AG, Herisau, Switzerland) as reported by Udenigwe et al. (2009). Flaxseed protein isolate (5%, w/v, protein basis) was suspended in deionized water in a reaction vessel equipped with a stirrer, heated to 37 °C and adjusted to the appropriate pH prior to the addition of one of the following enzymes; pepsin (pH 2.0–2.2), ficin (pH 7.0), trypsin (pH 8.0), papain (pH 6.5) or thermolysin (pH 8.0). The pancreatin reaction was conducted at temperature of 40 °C and pH 8.0. Each enzyme was added to the suspension at an enzyme-substrate ratio (E/S) of 1:100 (protein basis). Digestion was performed at the above conditions for 4 h; pH of the reaction mixture was kept constant by the pH-stat with 2 M NaOH except for the pepsin reaction. The Alcalase hydrolysis was carried out as previously reported (Omoni & Aluko,2006a, 2006b). After digestion, the enzyme was inactivated by immersing the reaction vessel in boiling water bath (95–100 °C) for 15 min and undigested proteins were precipitated by adjusting the pH to 4.0 with 2 M HCl followed by centrifugation at 14,941 *g* for 30 min. The supernatant containing the peptides was collected for further fractionation. Protein content of samples was determined by a modified Lowry's method (Markwell, Haas, Biebar, & Tolbert, 1978). The supernatant resulting from protein hydrolysis was passed through an Amicon stirred ultrafiltration cell set-up using a 1 kDa molecular weight cut-off (MWCO) membrane, and resulting permeate was collected. The FPH resulting from Alcalase digestion was passed through ultrafiltration membranes with MWCO of 5, 3 and 1 kDa to separate peptides of sizes 3–5, 1–3 and <1 kDa. To remove residual salts, the resulting permeates were dialysed for 48 h against deionized water at 4 °C using a 100 Da MWCO dialysis membrane and the retentates freeze-dried and stored at −20 °C until needed.

The cationic peptides in the <1 kDa permeate from the Alcalase FPH were separated as previously reported (Omoni & Aluko, 2006a) with some modifications. A sample solution (4 ml of 250 mg/ml) of the freeze-dried hydrolysate was loaded onto an SP-Sepharose High Performance XK 50/20 cation-exchange chromatography column connected to an AKTA Fast Protein Liquid Chromatography system (Amersham-GE Biosciences, Montreal, Canada). The column was pre-equilibrated with 1.5 column volume (CV) of 0.1 M ammonium acetate buffer, pH 7.5. After sample loading, the column was washed with 1.5 CV of 0.1 M ammonium acetate buffer to remove unbound peptides, followed by a gradient elution of the bound peptides using 0–50% 0.5 M ammonium carbonate, pH 8.8, in 0.1 M ammonium acetate buffer at a flow rate of 10 ml/min. The elution of peptides was monitored at 214 nm. Two major peaks (FI and FII) were observed and fractions within these peaks were pooled and concentrated by vacuum evaporation at 37 °C. Thereafter, the concentrated peptide solution was dialyzed for 48 h against deionized water at 4 °C. The content of

the dialysis bag was freeze-dried and stored at −20 °C until needed.

2.3. DPPH· scavenging assay

The scavenging activity of flaxseed peptide fractions against DPPH· was measured according to a previous method (Hou et al., 2001), which was slightly modified. Briefly, 160 μl of 100 μM DPPH· in methanol was mixed with 40 μl of flaxseed peptide fraction in a 96-well microplate for 20 min at room temperature under light protection. Thereafter, absorbance of the mixture was measured at 517 nm (*As*). Distilled water was used instead of the peptide fractions in blank experiments (*Ac*) whereas reduced glutathione (GSH) and BSA were used as positive and negative controls, respectively. The scavenging activity of peptide fractions was calculated using

$$\text{Activity} = \left[\frac{Ac - As}{Ac}\right] \times 100\% \quad (1)$$

Concentration-dependence of the scavenging properties of the active peptide fractions against DPPH· was also determined at four concentrations. The concentration of the samples that resulted in scavenging of 50% of DPPH· was calculated by non-linear regression and expressed as half maximal effective concentration (EC_{50}). The effect of buffers and pH on the scavenging activities of the peptide fractions against DPPH· was also investigated. The peptide fraction (60 μl at final concentration near the EC_{50}) was mixed with 40 μl of 0.1 M acetate buffers (pH 4.0, 4.5, 5.0, 5.5, 6.0), 0.1 M phosphate buffers (pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) and 0.1 M Tris–HCl buffers (pH 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0) in a 96-well microplate. DPPH· scavenging assay was performed as described above. The buffer solutions of different pH values were used instead of peptide solutions for control experiments. All assays were performed in triplicate.

2.4. Superoxide radical ($O_2^{\cdot-}$) scavenging assay

In this assay, ($O_2^{\cdot-}$) was generated from autoxidation reaction of pyrogallol (Gao, Yuan, Zhao, & Gao, 1998; Marklund & Marklund, 1974). Eighty microlitres of flaxseed peptide fraction of various concentrations was mixed with 80 μl of 50 mM Tris–HCl buffer (pH 8.3) containing 1 mM EDTA in a 96-well microplate followed by the addition of 40 μl of 1.5 mM pyrogallol in 10 mM HCl. The rate of ($O_2^{\cdot-}$)-induced polymerisation of pyrogallol (ΔA/min *s*) was measured as increase in absorbance at 420 nm for 4 min at room temperature. Tris–HCl buffer was used instead of peptide fraction in blank experiment (ΔA/min *c*) whereas GSH was used as positive control. All assays were performed in triplicate, and the scavenging activity of peptide fractions was calculated using

$$\text{Activity} = \left[\frac{\Delta A/\min c - \Delta A/\min s}{\Delta A/\min c}\right] \times 100\% \quad (2)$$

2.5. Hydroxyl radical (·OH) scavenging assay

Flaxseed peptide fractions were assayed for scavenging properties against ·OH generated from Fenton's reaction using ESR spectroscopy (Lin et al., 2008). Briefly, the reaction mixture (0.5 ml) contained 5 mM DMPO, 0.05 mM $FeSO_4$, 0.25 mM H_2O_2 and various concentrations of flaxseed peptide fractions. This solution was mixed and transferred to an ESR quartz cell. The cell was placed in the cavity of the ESR spectrometer and the relative intensity of the DMPO-OH spin adduct signal (*INTs*) was measured. Deionized water was used instead of sample solution for blank experiments (*INTc*). All ESR spectra were recorded at the ambient temperature (300 K) on a Bruker EMX-6/1 EPR spectrometer under the following conditions: centre field 345.4 ± 5.0 mT; microwave power 8 mW (9.416 GHz); modulation amplitude 5 G; modulation frequency 100 kHz; time constant 0.65 s; scan time 1.5 min. Scavenging activity of peptide fractions against ·OH was calculated as percentage using

$$\text{Activity} = \left[\frac{INTc - INTs}{INTc}\right] \times 100\% \quad (3)$$

2.6. Nitric oxide (NO) scavenging assay

NO was generated from SNP and measured as nitrite by the Griess reaction (Fiorentino et al., 2008). The assay mixture contained 5 mM SNP and 0.2 mg/ml flaxseed peptide fractions in 0.4 ml of 0.1 M phosphate buffer (pH 7.4). The assay mixture was incubated at 37 °C for 2 h. Thereafter, 0.1 ml of the reaction mixture was withdrawn and added onto a 96-well microplate followed by the addition of Griess reagent (50 μl of 0.1% N-1-napthylethylenediamine dihydrochloride in water, and 50 μl of 1% sulphanilamide in 5% phosphoric acid). The mixture was kept in the dark for 10 min at room temperature followed by measurement of the absorbance at 530 nm ($A_{530\ nm}$). Phosphate buffer was used instead of the peptide samples as blank. The scavenging activity of the samples against NO was expressed as percentage relative to $A_{530\ nm}$ of the blank experiment. All assays were performed in triplicate.

2.7. Determination of SSAO inhibition

SSAO inhibition assay was determined based on previous methods (Lin et al., 2008; Szutowicz, Kobes, & Orsulak, 1984) that were modified as follows. Bovine plasma (P-4639 reconstituted in 10 ml of deionized water) was used as a source of SSAO. The reaction mixture (200 μl) contained the following: 50 μl of 8 mM benzylamine, 50 μl of 200 mM phosphate buffer (pH 7.4), SSAO (2.53 units) and flaxseed peptide sample. The reaction mixture was incubated at 37 °C for 1 h followed by heating at 100 °C for 5 min to inactivate the enzyme and terminate the reaction. After cooling to room temperature and a brief centrifugation, 40 μl of the reaction mixture was withdrawn and added to a 160 μl solution containing 50 μl of 200 mM phosphate buffer (pH 7.4), 25 μl of 1 mM ABTS solution, 50 μl of horseradish peroxidase (5 μg/ml) and 35 μl of deionized water in a 96-well microplate. The rate of release of the reaction product was recorded as change in absorbance at 420 nm for 1 min (ΔA/min *s*). Deionized water was used instead of peptide sample solutions as blank (ΔA/min *c*). The percent (%) inhibition of SSAO activity was calculated using Eq. 2, and the concentration of the samples that resulted in inhibition of 50% of the activity of SSAO expressed as half maximal inhibitory concentration (IC_{50}). All assays were performed in triplicate.

2.8. LPS-induced NO production in RAW 264.7 macrophages

2.8.1. Cell culture and cell treatment

RAW 264.7 macrophages were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in DMEM (GibcoBRL, USA) supplemented with 10% fetal calf serum. The cells were seeded onto a 24-well culture plate at a density of 5×10^5 cells/ml and incubated at 37 °C in 5% CO_2 for 6 h prior to cell treatment. Thereafter, the cells were treated with only LPS, or LPS and PMB, or LPS and flaxseed peptides, in triplicate, and incubated at 37 °C for 24 h.

2.8.2. Nitrite quantification and cell viability assay

Nitrite formation was used to quantify NO production in the cell cultures. After 24 h incubation, 50 μl were withdrawn from each of the cell cultures and added onto a 96-well microplate followed by measurement of nitrite with Griess reagent as earlier described. Nitrite standard curve was prepared using 0–200 μM sodium nitrite. The% inhibition of NO production was calculated using the LPS-only treatment as 100%. The concentration of active flaxseed peptide fractions that suppressed 50% of NO production in the macrophages was calculated relative to control (no LPS) treatment and expressed as IC_{50}. Following the 24 h incubation, viability of the cells in all treatments was determined by MTT staining. The results were expressed as relative cell viability (%) using the blank (no LPS) treatment as reference (100%).

2.9. Statistical analysis

All data are reported as mean ± standard deviation of three separate determinations. Statistical significance of differences was evaluated by Student's *t*-test and Duncan's multiple range test ($p < 0.05$) using the Statistical Analysis Systems software (Statistical Analysis System, Cary, NC, USA).

3. Results and discussion

3.1. Peptide production and isolation

Despite their nutritionally rich amino acid profile, there is no report of large scale production of isolated flaxseed proteins for human consumption. This could be attributed to interferences of the polysaccharide gums of seed hulls during flaxseed protein isolation, which results in low protein yields (Oomah & Mazza, 1993). Several authors have attempted to improve the yield of isolated flaxseed protein using various methods including removal of the mucilage by de-hulling the flaxseed prior to protein isolation. Recently, Marambe et al. (2008) reported that laboratory de-oiling and subsequent removal of flaxseed mucilage using salt solution at 50 °C resulted in an isolated flaxseed protein with 80% protein content (N% × 6.25). However, the flaxseed meal utilised in our study was crushed and defatted for industrial applications without removal of the seed hull, and mucilage removal from the meal using dilute salt solution will solubilize its protein constituents. Thus, we developed a method to optimize the isolated protein yield from flaxseed meal by enzymatic removal of the seed mucilage using cellulase prior to protein isolation by alkaline solubilisation and acid-induced protein precipitation; this method provided an isolated flaxseed protein with 78.9% protein based on the modified Lowry protein assay.

The isolated flaxseed proteins were hydrolysed using seven proteases to generate potential bioactive peptides followed by fractionation into <1 kDa fractions using membrane ultrafiltration. The Alcalase hydrolysate was also fractionated into three fractions of molecular weight ranges 3–5, 1–3 and <1 kDa. The <1 kDa fraction of Alcalase FPH was further separated by fast protein liquid chromatography on a strong cationic exchange column, and this resulted in isolation of two cationic peptide fractions, FI and FII, with the latter possessing stronger cationic character as previously reported (Omoni & Aluko, 2006a). These peptide fractions were evaluated for antioxidant and potential anti-inflammatory properties. Since antioxidant activity may depend on factors such as the type of radical species involved in the reaction and the functionality of the antioxidant, it is appropriate to evaluate the antioxidant property of a sample using various assays. In this study, the potential antioxidant properties of flaxseed protein-derived peptide fractions were evaluated using free radical-scavenging assays against DPPH·, ($O_2^{\cdot-}$), ·OH and NO, as well as inhibition of SSAO activity.

3.2. Peptide-induced scavenging of free radicals

DPPH· is a stable N-containing free radical widely used in the primary assessment of antioxidant capacity of food. As shown in Fig. 1A, the flaxseed protein-derived peptides possess concentration-dependent scavenging activities against DPPH·; EC_{50} of the active fractions are shown in Table 1. The 1–3 kDa fraction of Alcalase FPH had the most potent activity better than those of the 3–5 kDa fraction and GSH. The papain-hydrolysed FPH fraction scavenged similar amount of DPPH· as GSH at twice the sample amount. The other peptide fractions and BSA did not scavenge DPPH·. Previous studies have reported the DPPH·-scavenging activities for various enzymatic food protein hydrolysates (Cumby, Zhong, Naczk, & Shahidi, 2008; Li, Han, & Chen, 2008; Li, Jiang, Zhang, Mu, & Liu, 2008; Sakanaka & Tachibana, 2006; Wang, Zhao, Zhao, & Jiang, 2007; Xie, Huang, Xu, & Jin, 2008). In this study, it could be observed that the release of DPPH·-scavenging peptides from flaxseed proteins depends in part on the specificity of the proteases used in hydrolysis. Moreover, it was also observed that the high molecular weight (HMW) peptide fractions had better activity in reducing DPPH·. Similarly, other studies have reported that the DPPH scavenging activities of food protein hydrolysates may depend on the size of their constituent peptides (Li, Han, et al., 2008; Li, Jiang, et al., 2008; Wang et al., 2007; Wu, Chen, & Shiau, 2003). Since DPPH·-scavenging reaction is a single electron transfer (SET) reaction (Huang, Ou, & Prior, 2005; Prior, Wu, & Schaich, 2005), results from this study suggest that the HMW peptide fractions contained more amino acid groups that could readily donate electrons to DPPH· when compared to the smaller peptides.

Furthermore, the DPPH· scavenging properties of flaxseed peptide fractions were observed to be dependent on both pH and

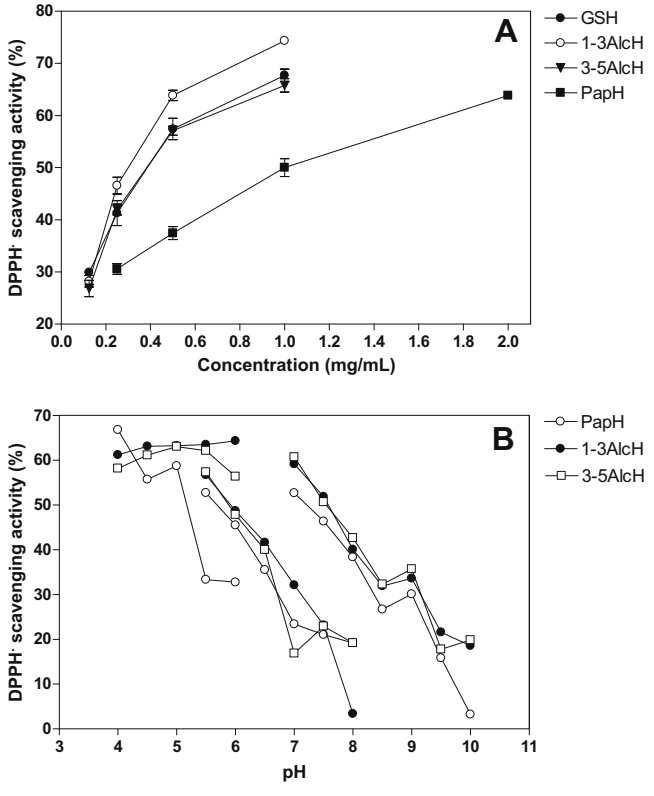

Fig. 1. (A) DPPH· scavenging activity of GSH (positive control), 1–3 kDa (1-3AlcH) and 3–5 kDa Alcalase FPH (3–5AlcH), and <1 kDa papain FPH (PapH). (B) The effects of pH and buffers on the DPPH· scavenging activity of the peptide fractions at concentration near their EC_{50} values; pH 4.0–6.0, 0.1 M acetate buffer; pH 5.5–8.0, 0.1 M phosphate buffer; pH 7.0–10.0, 0.1 M Tris–HCl buffer; Each point represents an average of three determinations.

Table 1
The EC_{50} (mg protein/ml) of flaxseed protein-derived peptide fractions in the scavenging of DPPH, ($O_2^{\cdot-}$) and ·OH, and IC_{50} (mg protein/ml) for *in vitro* inhibition of SSAO; results are expressed as mean ± standard deviation.

Sample	Radical-scavenging activity (EC_{50})			SSAO inhibition (IC_{50})
	DPPH·	($O_2^{\cdot-}$)	·OH	
Glutathione	0.385 ± 0.022	0.059 ± 0.009	0.0341 ± 0.0030[d]	–
FI	ND[a]	ND	<0.0800	>0.50
FII	ND	3.558 ± 0.117	ND	0.1321 ± 0.0218
1-3AlcH[b]	0.298 ± 0.019	1.719 ± 0.114	ND	0.0064 ± 0.0003
3-5AlcH[b]	0.382 ± 0.012	1.660 ± 0.060	ND	0.0069 ± 0.0006
PepH[c]	ND	ND	ND	>0.40
FicH[c]	ND	3.976 ± 0.017	0.2566 ± 0.0146	0.0926 ± 0.0052
TrypH[c]	ND	ND	0.09507 ± 0.0052	0.0269 ± 0.0019
PapH[c]	1.02 ± 0.083	3.944 ± 0.049	0.5053 ± 0.1272	0.1537 ± 0.0083
ThermoH[c]	ND	ND	0.0457 ± 0.0047	0.0377 ± 0.0045
PancH[c]	ND	ND	0.0682 ± 0.0048	0.1277 ± 0.0076

[a] ND, not determined because of low or nil activity.
[b] Ultrafiltration permeate from alcalase FPH: 1–3 kDa, 1–3AlcH; 3–5 kDa, 3–5AlcH.
[c] Ultrafiltration permeate (<1 kDa) from FPH produced with pepsin, PepH; ficin, FicH; trypsin, TrypH; papain, PapH; thermolysin, ThermoH; pancreatin, PancH.
[d] Concentration-dependence was lost after 0.04 mg/ml glutathione (GSH).

buffer (Fig. 1B). These results show that peptide fractions exhibited better radical-scavenging activity in acetate buffer than in phosphate and Tris–HCl buffers; similar DPPH·-scavenging activity patterns were observed for all peptide samples in the different buffers except for the activity of LMW peptides from papain FPH at pH 5.5 and 6.0 in acetate buffer. Using Tris–HCl buffer, it was observed that the peptide fractions exhibited their best activity at pH 7.0, and this was lost with increase in pH even at 8.0, which is similar to the condition presently used in most DPPH· scavenging assays. Furthermore, it was observed that phosphate buffer is not suitable for this assay since the peptide fractions lost up to 35% of their DPPH· scavenging properties; the most potent DPPH· scavenger in this study showed almost no activity in phosphate buffer at pH 8.0. These results are in agreement with previously reported evidence that pH affects the DPPH·-scavenging properties of antioxidants (Liu, Wu, et al., 2007; Lin et al., 2008). In the SET DPPH·-scavenging reaction, as suggested by Lin et al. (2008) for geraniin, pH conditions might affect ionisation potential and electron transfer capacity of peptides, which could explain the different activities observed under different pH. However, due to the stability of DPPH·, some potential antioxidants may not show scavenging activity within the assay duration (Huang et al., 2005). Moreover, DPPH· is not functionally similar to highly reactive peroxyl radicals that cause oxidation of biological macromolecules (Huang et al., 2005; Prior et al., 2005), which raises questions about the physiological relevance of DPPH·-scavenging antioxidants. Based on these and other limitations, highly reactive and physiologically-relevant free radicals were used to evaluate antioxidant properties of the flaxseed peptide fractions.

In alkaline solution, ($O_2^{\cdot-}$) induces pyrogallol autoxidation leading to the formation of conjugation products that can be detected at 420 nm; reducing compounds inhibit this reaction by acting as ($O_2^{\cdot-}$) scavengers; EDTA is used as metal chelator to eliminate the effects of interfering metal ions (Gao et al., 1998; Marklund & Marklund, 1974). The ($O_2^{\cdot-}$) generated from autoxidation of pyrogallol was used to evaluate the antioxidant property of the flaxseed peptides, which showed concentration-dependent activity (Fig. 2A). As observed in the DPPH· assay, activity of the peptide fractions in scavenging ($O_2^{\cdot-}$) differed depending on the protease used in protein hydrolysis and MW ranges of the fractions; the higher MW peptide fractions showed stronger scavenging activity with lower IC_{50} (Table 1). Hydrolysis with ficin and papain yielded <1 kDa peptides with moderate and similar ($O_2^{\cdot-}$) scavenging activities. Moreover, purification of the <1 kDa fraction of Alcalase FPH using a cation-exchange column yielded cationic peptide fraction

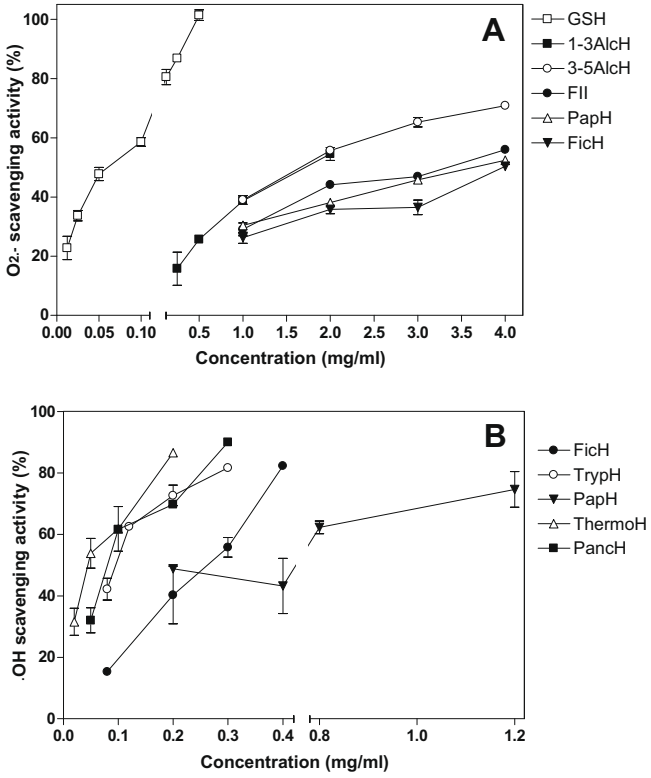

Fig. 2. (A) Flaxseed peptide fractions and GSH with dose-dependent ($O_2^{\cdot-}$) scavenging activities; see Table 1 for abbreviations. (B) The scavenging activities of peptide fractions against ·OH measured by electron-spin resonance spectroscopy.

II, which also scavenged ($O_2^{\cdot-}$). The ($O_2^{\cdot-}$)-scavenging activities of these peptide fractions were observed to be weaker than the activity of GSH, which completely scavenged ($O_2^{\cdot-}$) at 0.5 mg/ml. In comparison, the ($O_2^{\cdot-}$)-scavenging of Alcalase alfalfa leaf protein hydrolysate (0–0.9 mg/ml) was reported to range from 0% to 67% (Xie et al., 2008) and that of Alcalase chickpea hydrolysate fractions (2.0 mg/ml) from 35–69% (Li, Jiang, et al., 2008). In addition, egg yolk proteins hydrolyzed with proteinase from *Bacillus* sp. scavenged about 5–90% ($O_2^{\cdot-}$) at 0.0313–0.5% protein (Sakanaka & Tachibana, 2006). In this study, the <1 kDa permeate of trypsin and thermolysin FPH as well as Alcalase cationic peptide fraction I showed meagre antioxidant properties by scavenging only 12.6,

17.5 and 19.4% ($O_2^{\cdot-}$), respectively at 2 mg protein/ml whereas both the pepsin and pancreatin treatments yielded LMW peptides that could not scavenge ($O_2^{\cdot-}$).

The ·OH-scavenging property of the flaxseed peptides was evaluated using an assay similar to the physiological system whereby ·OH was generated by reaction of Fe^{2+} and H_2O_2 (Fenton reaction). The ·OH produced in the reaction was spin-trapped with DMPO, and the intensity of the DMPO-OH adducts measured using ESR spectrometer. A decrease in intensity or disappearance of the DMPO-OH signal is an indication of the ·OH scavenging activity of antioxidants (Li et al., 2004). It was observed in this study that most of the FPH fractions were capable of reducing the intensity of DMPO-OH signals at various protein concentrations. Fig. 2B shows that there was a linear relationship between ·OH-scavenging activity and the amount of peptide samples in the assay. Unlike the antioxidant properties observed in the DPPH· and ($O_2^{\cdot-}$) assays, the LMW flaxseed peptides showed potent ·OH-scavenging activities except for the pepsin hydrolysate (Table 1). The thermolysin, pancreatin and ficin hydrolysates scavenged 86.5, 89.9 and 82.3% ·OH, respectively at 0.2, 0.3 and 0.4 mg/ml, respectively. The least cationic peptide fraction from Alcalase hydrolysate (FI) also displayed potent ·OH-scavenging activity with inhibition of 55.6, 76.7 and 81% ·OH at 0.08, 0.12 and 0.2 mg/ml, whereas the stronger cationic peptides (FII) did not. This observation is inversely correlated with their observed activity in the ($O_2^{\cdot-}$)-scavenging assay. Moreover, the ·OH-scavenging properties of these flaxseed peptide fractions reported in this paper are better than the recently reported activity of crude hydrolysates from Flavourzyme digestion of flaxseed proteins (IC_{50}, 1.56–3.06 mg/ml) (Marambe et al., 2008). Previous studies have also reported that, using different spectrophotometric assay systems, the peptide fractions from Alcalase chickpea protein hydrolysate (1.5 mg/ml) scavenged 38% to 81% ·OH (Li, Jiang, et al., 2008) whereas treatment of egg yolk protein with *Bacillus* sp. proteinase yielded hydrolysates that scavenged 74% of ·OH at 0.5 mg/ml (Sakanaka & Tachibana, 2006). In addition, a recent study reported that whey protein hydrolysate and its peptide fractions showed moderate antioxidant properties against ·OH and other free radicals when measured by ESR spectroscopy (Peng, Xiong, & Kong, 2009) similar to the method used in this present study. Contrary to the results in the DPPH· and ($O_2^{\cdot-}$) assays, the HMW flaxseed peptide fractions did not show ·OH-scavenging activity. It should be noted that this assay method has a limitation as it is almost impossible to determine whether the activity of antioxidants is due to scavenging of ·OH or chelation of Fe^{2+}, since most potential antioxidants are also good metal chelators that might decrease ·OH production (Huang et al., 2005), thus reducing the intensity of the DMPO-OH adduct. However, Peng et al. (2009) recently reported that the ·OH-scavenging activity observed in their study for whey protein hydrolysate fractions could be attributed to the hydrolysates, which they reported to possess poor Fe^{2+} chelating activities.

3.3. Peptide-induced inhibition of SSAO

The *in vitro* SSAO inhibitory activities of the flaxseed protein-derived peptide fractions are shown in Fig. 3; all the peptide samples evaluated in this study exhibited concentration-dependent inhibition of SSAO activity which was also dependent on size of the peptides. The HMW peptides from Alcalase hydrolysate potently inhibited SSAO activity at low concentrations. Moreover, the LMW peptide fractions as well as Alcalase cationic peptide FII also inhibited SSAO activity. The weakest inhibitory activity was observed for the pepsin hydrolysate LWM fraction and the Alcalase cationic peptide FI. The IC_{50} values for the peptide fractions in SSAO inhibition are shown in Table 1. To the best of our knowledge, this work is the first to report the activity of enzymatic food protein

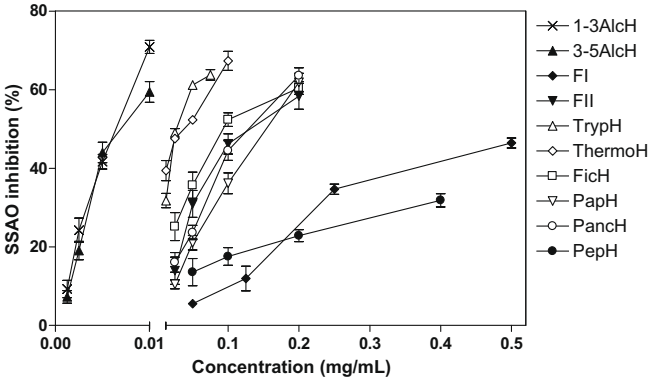

Fig. 3. Inhibition of SSAO by flaxseed peptide fractions; inhibitory activity of peptides was dependent on size and nature of the peptides; thus, the higher MW peptides showed the best activity.

hydrolysates in inhibition of SSAO activity. Since physiological SSAO reaction generates H_2O_2 and reactive aldehydes that play important roles in pathogenesis of disease conditions, its inhibition by these flaxseed peptide fractions may constitute a step in treatment of inflammation and other related diseases.

3.4. Peptide-induced inhibition of in vitro and ex vivo NO production

The cellular toxicity of NO has been associated with its reaction derivatives especially $ONOO^-$, which could lead to DNA fragmentation and protein structure modification. Thus, NO scavengers could lower the risk of cellular and tissue damages associated with excessive NO production. Fig. 4 shows the *in vitro* scavenging activities of the flaxseed peptide fractions against NO; at 0.2 mg/ml, the peptide fractions showed weak NO scavenging properties. Both the thermolysin and pancreatin FPH fractions gave the strongest activity by scavenging 27.1% and 35.1% NO, respectively. The other peptide fractions scavenged $\leqslant$20.3% NO at the same concentration. Moreover, in order to evaluate their anti-inflammatory properties, the flaxseed peptide samples were screened for effects on LPS-induced NO production in macrophages. The treatment of RAW 264.7 macrophages with LPS resulted in increased NO production in the cell culture (Fig. 5). The addition of PMB to the cell culture prior to LPS activation resulted in normalized NO production, since PMB binds and inactivates LPS. On treatment of the macrophage

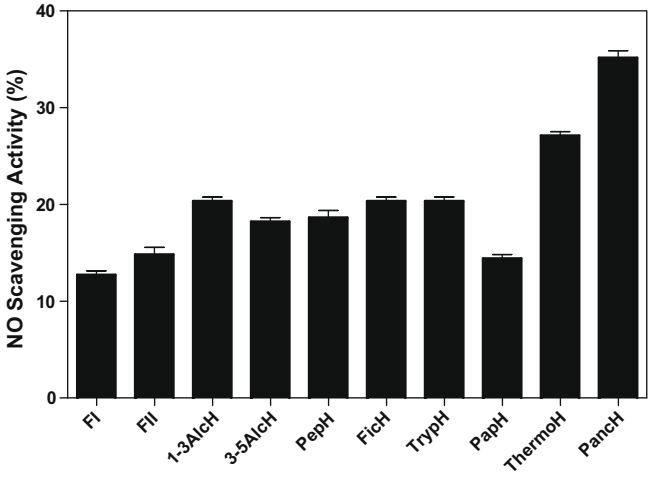

Fig. 4. The *in vitro* scavenging activities of 0.2 mg/ml flaxseed protein-derived peptide fractions against NO produced from SNP.

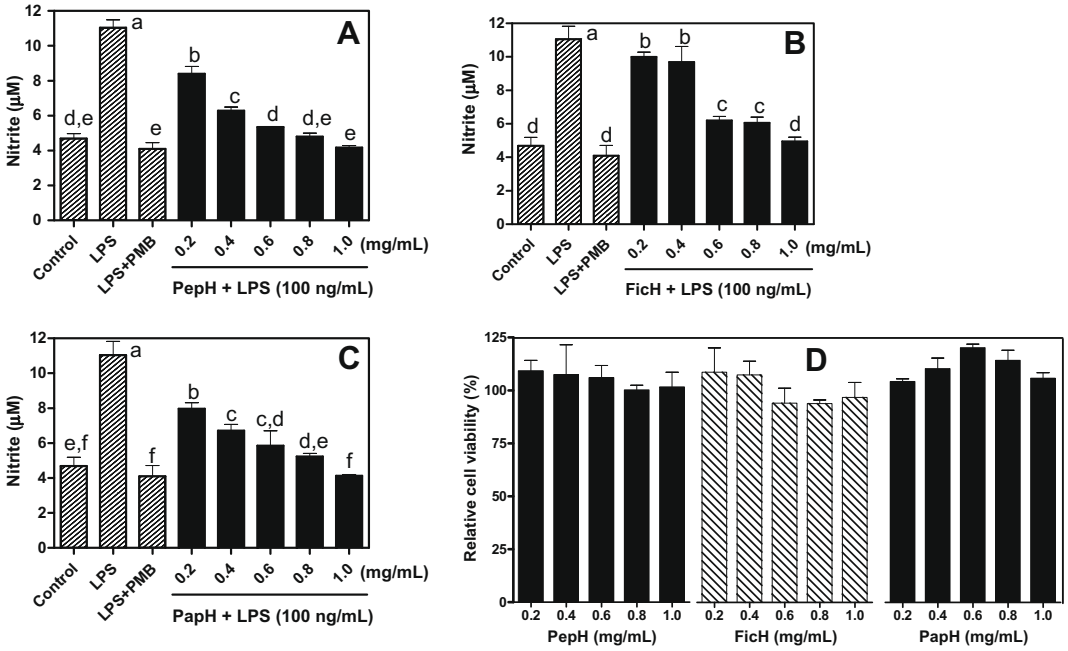

Fig. 5. Effects of flaxseed protein-derived (A) PepH, (B) FicH and (C) PapH on LPS-induced NO production in RAW 264.7 cells; the cells were treated as follows: no LPS (control); LPS (100 ng/ml) only; LPS (100 ng/ml) plus PMB (50 μg/ml); and LPS (100 ng/ml) plus 0.2–1.0 mg protein/ml flaxseed peptide fraction. PMB was used as positive control. The treated cells were incubated at 37 °C and 5% CO_2 for 24 h followed by determination of cellular production of NO and cell viability. Each bar represents mean of triplicate determinations; bars with the same letter within each graph are not significantly different at $p = 0.05$ (D) Viability of RAW 264.7 cells in the presence of the peptide fractions, which showed no toxicity against the growth of the macrophages.

cultures with LPS and the various flaxseed peptides, the pepsin, ficin and papain FPH fractions were observed to exhibit concentration-dependent inhibition of LPS-induced NO production in the macrophages with IC_{50} values of 0.250, 0.504 and 0.215 mg protein/ml, respectively (Fig. 5A–C). At 1 mg protein/ml, the pepsin and papain FPH fractions completely inhibited the endotoxin-induced NO production to the basal level observed for control and PMB treatments. Moreover, these flaxseed peptide fractions did not show cytotoxicity against the cells within the range of concentrations studied using the MTT staining assay (Fig. 5D). It is noteworthy that, despite showing the best *in vitro* NO scavenging activities (Fig. 4), the thermolysin and pancreatin FPH fractions did not affect concentrations of NO in the cell cultures when compared to the LPS-only treatment (data not shown). Thus, the ability of the flaxseed peptides to scavenge NO *in vitro* is not correlated with their inhibitory activities against NO production or their potential NO scavenging properties in the LPS-treated culture. It is possible that the bioactive peptides present in thermolysin and pancreatin FPH were metabolized to inactive fragments by the macrophages. It could also be suggested that the active flaxseed peptide fractions may have altered the pathway for NO synthesis in the macrophages. It has been shown that the activity of potential therapeutic agents in inhibition of NO production could be through inhibition of transcription factor NF-κB activation and subsequent inhibition of iNOS mRNA and protein expressions in macrophages (Chen et al., 2001; Ho & Lin, 2008; Pan et al., 2008). To the best of our knowledge, there is limited information in the literature regarding the effects of enzymatic food protein hydrolysates on endotoxin-induced NO production in cell cultures. A previous study reported that a hydrolyzed casein diet fed to young diabetes-prone BB rats led to restoration of NO production to basal level in interleukin-1β-induced overproduction of NO in the pancreatic islets (Malaisse et al., 2000). Recently, lunasin, a naturally occurring oligopeptide from soybean was reported to possess potential *in vitro* anti-inflammatory activity due to its inhibitory effects on the production of NO and prostaglandin E_2 through the inhibition of iNOS and cyclooxygenase-2 protein expressions, respectively, in LPS-activated RAW 264.7 macrophages (Dia, Wang, Oh, de Lumen, & Gonzalez de Mejia, 2009). However, yam dioscorin had been shown to exhibit immunomodulatory effects partly by inducing NO productions in macrophages in the absence of LPS (Liu, Shang, Wang, Hsu, & Hou, 2007). Moreover, it was also observed in this present study that the flaxseed protein-derived peptide fractions did not induce excessive production of NO in the macrophages when LPS was eliminated with PMB (data not shown). Furthermore, all the flaxseed protein-derived peptide fractions in this study were also evaluated for cytotoxicity against two cancer cell lines, HL-60 (human promyelocytic leukemia, ATCC CCL-240) and MCF7 (human breast adenocarcinoma, ATCC HTB-22) using the MTT staining assay. The flaxseed peptide fractions did not show cytotoxicity against these cancer cell lines at 0.2 mg protein/ml after 24 h of incubation (data not shown).

4. Conclusions

Enzymatic hydrolysis of flaxseed proteins by various proteases resulted in the release of bioactive peptides with antioxidant and potential anti-inflammatory properties. Bioactive properties of the flaxseed peptide fractions were dependent on catalytic specificity of the proteases as well as the size of peptides in the resulting protein hydrolysate fractions. Papain treatment released multifunctional flaxseed peptides with MW less than 1 kDa that exhibited comparatively moderate biological activities in all the *in vitro* antioxidant assays evaluated in this study. On the other hand, pepsin-catalysed hydrolysis yielded poor free radical-scavenging <1 kDa flaxseed peptides but with excellent restoration of LPS-induced NO production to basal level in macrophages. The other <1 kDa flaxseed peptide fractions showed potent or moderate activities in scavenging free radicals; however, the <1 kDa cationic flaxseed peptide fractions did not exhibit good antioxidant

property except the least cationic fraction (FI) that potently scavenged ·OH. Overall, the HMW peptides (1-3 and 3-5 kDa) showed better antioxidant properties than the LMW peptides except in scavenging ·OH and inhibition of NO production in macrophages. These bioactive properties could encourage increased value-added utilisation of flaxseed meal proteins for the formulation of therapeutic products. Evaluation of the effects of the flaxseed protein-derived peptides in appropriate animal disease models is required to confirm some of the preliminary results obtained in this work.

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