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ARTICLE

Antioxidant and Angiotensin Converting Enzyme-Inhibitory Properties of a Flaxseed Protein-Derived High Fischer Ratio Peptide Mixture

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Hydrolysis of flaxseed proteins using thermolysin and pronase followed by mixing with activated carbon, centrifugation and filtration yielded a filtrate (peptide mixture) with a Fischer ratio (branched-chain amino acids/aromatic amino acids) of 23.65 and a phenylalanine + tyrosine content of 1.11%. Gel permeation chromatography showed that the flaxseed peptide sample contained mainly low molecular weight peptides (<4 kDa). The high Fischer ratio peptide sample exhibited antioxidant property by scavenging 2,2-diphenyl-1-picrylhydrazyl radical, superoxide radical, hydroxyl radical, and also by protecting linoleic acid from oxidation. In addition, the peptide mixture showed potential antihypertensive properties by inhibiting angiotensin I-converting enzyme in a mixed-type inhibition pattern. Protein hydrolysates with Fischer ratio higher than 20 and phenylalanine + tyrosine content lower than 2% have been used to treat patients with hepatic encephalopathy; thus, this multifunctional flaxseed peptide mixture could be used to formulate food products with multiple human health benefits during liver diseases, oxidative stress and hypertension.

KEYWORDS: Flaxseed protein; Fischer ratio; activated carbon; branched-chain amino acids; aromatic amino acids; antioxidant; angiotensin converting enzyme

INTRODUCTION

Acute and chronic liver failure is associated with alterations in protein metabolism, which can lead to loss of muscle protein mass. Plasma amino acid imbalance has been found in patients with liver diseases, and this is characterized by elevated levels of aromatic amino acids (AAA; tyrosine and phenylalanine) and methionine, and decreased levels of branched-chain amino acids (BCAA; leucine, isoleucine and valine) (*1*). The ratio of plasma BCAA to AAA is termed Fischer ratio and is used to measure abnormal amino acid metabolism during liver disease, and also in clinical nutrition for the formulation of amino acid products for therapeutic purposes. In human beings, the normal plasma Fischer ratio is 3.5−4.0; this value decreases to <2.5 in liver diseases, <1.2 in hepatic coma and could drop to below 0.8 in profound coma (*2*). Clinical nutrition approaches toward treatment of liver diseases involve feeding patients with BCAA-enriched formulas (*3*−*6*) or protein hydrolysates with Fischer ratio higher than 20 and phenylalanine + tyrosine content lower than 2% (*7*). BCAAs have been shown to reduce muscle wasting and plasma concentrations of AAA with concomitant improvement in encephalopathy (*8*). The therapeutic effect of BCAA is due to the fact that they are preferentially taken up by the muscle, inhibit the transport of AAA across the blood−brain barrier, and are available for peripheral metabolism in advanced liver disease (*1*). In addition, some athletes also use BCAA as a natural way of improving muscle mass and strength (*9*).

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A number of studies have reported successful production of high Fischer ratio peptide mixtures by enzymatic hydrolysis of food proteins and subsequent processing using activated carbon and gel filtration chromatography (*10*−*14*). In clinical nutrition, peptides are preferred over individual amino acids due to the fact that short peptides have higher rates of intestinal absorption and the osmotic pressure of peptides is lower than that of the corresponding free amino acids (*15*, *16*). The recommended criteria for selecting the starting food protein that will yield a high Fischer ratio peptide mixture include high amounts of BCAA, low amounts of AAA (*1*) and availability of the raw material.

Flaxseed (*Linum usitatissimum*) is a readily available oilseed predominantly produced in the Prairie regions of Canada, and a major dietary source of α-linolenic acid, dietary fibers and lignans (*17*). These flaxseed components have been reported to possess various physiological activities relevant to human health sustenance especially in cardiovascular disease prevention, cancer and diabetes (*17*). However, the protein components of flaxseed have been scarcely explored for nutritional and therapeutic applications. These proteins constitute a major part of defatted flaxseed meal, which results from industrial flaxseed crushing and oil extraction, and is comparable in nutritional quality and amino acid composition to other high quality proteins, e.g. soy protein (*17*, *18*). Moreover, previous studies have reported that enzymatic hydrolysis of flaxseed proteins afforded low molecular weight (MW) peptides that possess antioxidant and anti-inflammatory properties in cell cultures (*19*) as well as *in vitro* antihypertensive properties (*20*). Flaxseed proteins contain high

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levels of BCAA, with 50, 71, and 56 mg/g of isoleucine, leucine and valine (*21*), respectively. Thus, through careful choice of enzymes and processing conditions, flaxseed proteins have the potential to be converted into protein hydrolysates enriched with BCAA.

Food protein-derived peptides possess physiological therapeutic roles such as antioxidant and antihypertensive properties. Endogenous and exogenous reactive oxygen species and free radicals are implicated in the initiation and progression of human disease conditions (*22*). The amount of these reactive species is controlled by endogenous antioxidants until it reaches a level when the antioxidants are overwhelmed, a state known as oxidative stress. This condition can be surmounted by the consumption of foods rich in antioxidants. The renin−angiotensin system, which controls blood pressure in human beings, has been targeted for the treatment of hypertension through the inhibition of angiotensin I-converting enzyme (ACE) and renin. ACE inhibitors are thought to possess the ability to lower blood pressure in hypertensive subjects and animals (*23*). Food protein hydrolysates and constituent peptides with multifunctional bioactive properties against these disease targets are desirable for the formulation of food products with human health benefits in multiple physiological and disease conditions. Therefore, a BCAA-enriched product that possesses other bioactive properties could, in addition to the positive effects on liver disease, be potentially useful as a single agent to provide simultaneous relief from other associated disease symptoms.

The objectives of this project were to produce a high Fischer ratio enzymatic flaxseed protein hydrolysate and determine the ability of the hydrolysate to scavenge free radicals and inhibit *in vitro* oxidative reactions and activities of ACE and renin.

MATERIALS AND METHODS

Materials. Thermolysin (from *Bacillus thermoproteolyticus rokko*), Alcalase (from *B. licheniformis*), ficin (from fig tree latex), papain (from papaya latex), activated carbon, pyrogallol (1,2,3-trihydroxybenzene) and *N*-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine (FAPGG) were purchased from Sigma Chemicals (St. Louis, MO). Pronase (from *Streptomyces griseus*) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). All other chemical reagents were of analytical grade.

Enzymatic Hydrolysis of Flaxseed Protein. Flaxseed protein isolate (FPI) was prepared from defatted flaxseed meal as previously described (*20*). The overall approach involved hydrolysis of flaxseed proteins with thermolysin followed by treating the resulting protein hydrolysate with papain, ficin, alcalase or pronase. FPI (5% w/v) was suspended in distilled water; the slurry was adjusted to pH 7.5 with 1 M Na_2CO_3 solution and to a temperature of 37 °C. Thereafter, protein hydrolysis was initiated by adding thermolysin at an E/S ratio of 1:100 (based on protein content). During hydrolysis, the reaction mixture was maintained at pH 7.5 by adding 1 M Na_2CO_3 solution using a pH stat instrument (Metrohm Titrando, Herisau, Switzerland). After 5 h, the enzymatic reaction was terminated by adjusting to pH 4.0 using 1 M HCl. The temperature and pH of the reaction mixture was adjusted appropriately and subjected to another hydrolysis using one of these proteases: papain (pH 6.5, 37 °C), ficin (pH 7.0, 37 °C), alcalase (pH 8.0, 50 °C) and pronase (pH 7.4, 40 °C). These enzymes were separately added to the resulting hydrolysate for further hydrolysis at E/S ratio of 1:100 (based on protein content); pH was appropriately maintained with 1 M Na_2CO_3 solution during proteolysis. After 5 h, proteolysis was stopped by adjusting to pH 4.0 using 1 M HCl. The resulting flaxseed protein hydrolysate (FPH) was collected by centrifugation at 15000*g*, freeze-dried and stored at −20 °C until used. The protein content of the resulting powder was determined by a modified Lowry protein assay method (*24*).

Adsorption of Flaxseed Protein Hydrolysate onto Column-Packed Activated Carbon. To separate the BCAA-containing peptides from the AAA-containing peptides, each of the freeze-dried FPH was dissolved in deionized water at 10 mg/mL and the solution was adjusted to pH 2.5 using 0.5 M HCl (*14*) followed by filtration using Whatman No. 1 filter paper. Thereafter, the resulting acidic filtrate was passed through a column packed with activated carbon (9.5 × 80 mm) and sample was eluted using a single-channel Peristaltic pump model P-1 (GE Healthcare, Montreal, PQ, Canada). The unbound peptides (BCAA-rich) were collected in the flow-through solution and labeled as FPH-col. The column was regenerated for subsequent fractionation by eluting with absolute ethanol to remove bound peptides (AAA-rich) followed by washing with distilled water. Peptide elution was monitored using a spectrophotometer by measuring absorbance at 220, 260, and 280 nm. The collected peptide solutions were freeze-dried and stored at −20 °C until used.

Adsorption of Flaxseed Protein Hydrolysate onto Activated Carbon by Simple Mixing. The AAA-containing peptides and free AAA were also removed from the FPH samples by mixing a solution containing 10 mg/mL of FPH (previously adjusted to pH 2.5 using 0.5 M HCl) with various amounts of activated carbon (10, 25, 50, 75, 100, and 150 mg) in a centrifuge tube for 10 min at 22−23 °C. Thereafter, the mixture was centrifuged at 15000*g* for 30 min and the supernatant (contains BCAA-rich peptides) were filtered through a Whatman No. 1 filter paper to completely remove traces of activated carbon. The filtrate (FPH-mix) was freeze-dried and stored at −20 °C until used. The levels of peptides and AAA in the FPH-mix peptide product were initially estimated using a spectrophotometric absorbance at 220 and 260 + 280 nm, respectively.

Amino Acid Analysis. An HPLC system was used to determine the amino acid profiles after samples were hydrolyzed with 6 M HCl as previously reported (*25*). The cysteine and methionine contents were determined after performic acid oxidation (*26*) and tryptophan content was determined after alkaline hydrolysis (*27*). The Fischer ratio was calculated as the ratio of BCAA to AAA.

Gel Permeation Chromatography. Molecular weight distribution of the peptides present in the FPH-mix that had the highest Fischer ratio was estimated by gel filtration chromatography as previously reported (*28*) with some modifications. The peptide sample (10 mg/mL) was filtered using a 0.2 μm membrane, and 100 μL of the solution was injected onto a Superdex 75 100/300 L column connected to an AKTA fast protein liquid chromatography system (GE Healthcare, Montreal, QC, Canada). The column was equilibrated with 1 column volume (23.5 mL) of 50 mM phosphate buffer containing 0.15 M NaCl (pH 7.0) prior to loading the sample. The peptides were eluted from the column using a flow rate of 0.75 mL/min, and peaks were detected at 214 nm. The column was calibrated using the following standards: cytochrome *c* (12.3 kDa), aprotinin (6.5 kDa), pepstatin A (685.9 Da), glutathione (307.33 Da) and glycine (75 Da); blue dextran was used to determine the column void volume. The estimated molecular weights of the eluted peaks were determined from a plot of the elution volume versus the $\log_{10}$ MW of the standards.

Antioxidant Assays. The ability of the FPH-mix peptide sample with the highest Fischer ratio to scavenge DPPH radical was evaluated as previously reported (*19*). Superoxide ($O_2^{\bullet-}$) scavenging assay was also evaluated using the pyrogallol autoxidation method as previously described (*19*). Briefly, 80 μL of the peptide solution 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 $O_2^{\bullet-}$-induced polymerization of pyrogallol (ΔA/min) was measured as increase in absorbance at 420 nm for 4 min at room temperature. Tris-HCl buffer was used to replace the peptide solution in control experiment whereas glutathione was used as positive control. $O_2^{\bullet-}$ scavenging activity (%) was calculated as $\{[(\Delta A/\text{min})_c - (\Delta A/\text{min})_s]/(\Delta A/\text{min})_c\} \times 100$, where c and s represent control and sample, respectively.

In addition, $^{\bullet}$OH scavenging activity of the high Fischer ratio FPH-mix sample was assayed based on a method described by Li et al. (*29*). Peptide samples (50 μL in 0.1 M sodium phosphate buffer, pH 7.4) were mixed with 50 μL of 3 mM 1,10-phenanthroline (in phosphate buffer) and 50 μL of 3 mM $FeSO_4$ (in water) in a 96-well microplate. Thereafter, 50 μL of 0.01% aqueous H_2O_2 was added to initiate the Fenton reaction. The reaction mixture was covered and incubated at 37 °C for 1 h and the absorbance measured at 536 nm $[(A_{536nm})_s]$. A control experiment was also conducted by replacing the peptide sample with buffer $[(A_{536nm})_c]$. The $^{\bullet}$OH scavenging activity (%) was calculated as $\{[(A_{536nm})_c - (A_{536nm})_s]/(A_{536nm})_c\} \times 100$.

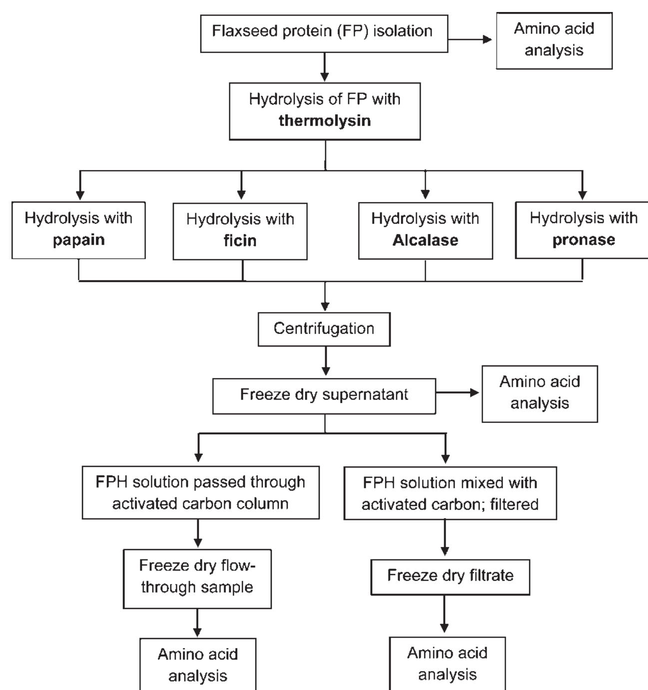

Figure 1. Flowchart for production of BCAA-enriched high Fischer ratio mixture by enzymatic hydrolysis of isolated flaxseed protein and activated carbon treatment.

The ability of the FPH-mix to inhibit linoleic acid (LA) oxidation was also investigated as previously described (*29*) with modifications. LA (1 mL of 50 mM dissolved in 99.5% ethanol) was mixed with 0.5 mL of a solution of different concentrations of the peptide sample (final concentrations of 5, 2.5, and 1.25 mg/mL of 0.1 M phosphate buffer, pH 7.0) or glutathione (final concentration of 1.25 mg/mL). The mixture was kept at 60 °C in darkness for 6 days. An aliquot of 0.1 mL of the sample solution was withdrawn after every 24 h, and mixed with 4.7 mL of 75% aqueous ethanol, 0.1 mL of ammonium thiocyanate (30% w/v) and 0.1 mL of 20 mM $FeCl_2$ (dissolved in 1 M HCl). The absorbance of the mixture was measured at 500 nm after 3 min incubation at room temperature.

ACE and Renin Inhibition Assays. The potential antihypertensive property of the FPH-mix sample with highest Fischer ratio was determined based on its ability to inhibit the activities of ACE and renin *in vitro*. At various concentrations of the peptide sample, ACE and renin inhibitory assays were carried out as previously described using FAPGG as ACE substrate and Arg-Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(Dabcyl)-Arg as renin substrate (*20*). In addition, the kinetics of inhibition of ACE was also studied to determine the mode of the peptide-induced ACE inhibition. Kinetics parameters (K_m and V_{max}, and their apparent values in the presence of the peptide sample) were estimated from nonlinear regression fits of the kinetics data to the Michaelis−Menten equation using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA). The catalytic efficiency of ACE in the presence and absence of the peptide sample was calculated as V_{max}/K_m. All the kinetics experiments were conducted in duplicate.

Statistical Analysis. Except where indicated, data were collected in triplicate and subjected to one way analysis of variance using Statistical Analysis System Software (SAS version 9.2, SAS Institute, Cary, NC). Significant differences were determined by Duncan's multiple range test and accepted at $p < 0.05$.

RESULTS AND DISCUSSION

Enzymatic Flaxseed Protein Hydrolysis. Figure 1 shows the procedure used for hydrolyzing the flaxseed protein and processing of the resulting FPH. The aim of the enzymatic hydrolysis of flaxseed protein in this study was to produce peptides with low contents of AAA and generate low-MW peptides for efficient intestinal absorption. Thermolysin was chosen for the first hydrolysis step because of its specificity in cleaving proteins at the N-terminal regions of hydrophobic amino acids, phenylalanine, tyrosine, leucine, isoleucine and valine. In order to release the N-terminal AAA, the resulting protein hydrolysate was subjected to another controlled hydrolysis using other specific proteases, papain and ficin. Papain has a wide specificity but preferentially cleaves the X_1-X_2 peptide bond in Phe$-X_1-X_2$, where X_1 and X_2 are any amino acids, thereby releasing a Phe-containing dipeptide into the solution. A combination of thermolysin and papain has been previously used to produce high Fischer ratio peptide mixtures (*14*, *30*). Ficin, a papain-like protease, was used as a control since it has a wide specificity in cleaving the carboxyl side of 10 different amino acid residues, including valine and tyrosine. Moreover, the highly nonspecific proteases were used to produce peptides of shorter chain length that could be easily absorbed in the intestine. Alcalase is an endopeptidase whereas pronase possesses both endopeptidase and exopeptidease activity and can generate low molecular weight peptides and free amino acids from native and denatured proteins and large peptides. Thus, pronase can cleave the N-terminal AAA resulting from the thermolysin activity. The protein contents of the FPH samples were 81% (papain), 85.2% (ficin), 66.3% (alcalase) and 60.7% (pronase) based on Lowry assay. The observed low protein contents of the FPH generated with pronase may be due to their exopeptidase activity, which released free amino acids that were not detected by the Lowry assay.

Table 1. Fischer Ratios (BCAA/AAA) and Phe + Tyr Contents of Enzymatic Flaxseed Protein Hydrolysates (FPH) Passed through a Column Packed with Activated Carbon (FPH-col) or Mixed with Activated Carbon Inside a Centrifuge Tube (FPH-mix)[a]

	papain	ficin	Alcalase	pronase
		Fischer Ratio		
FPH	1.84	1.60	1.67	1.72
FPH-col	3.05	2.92	2.53	4.04
FPH-mix	2.80	6.21	4.26	23.65
		Phe + Tyr (%)		
FPH	8.29	8.29	8.06	8.30
FPH-col	5.28	2.75	3.71	3.55
FPH-mix	5.06	5.03	4.95	1.11

[a] BCAA = branched-chain amino acids; AAA = aromatic amino acids.

Activated Carbon Treatments, Amino Acid Profiles and Fischer Ratios. The FPH was passed through a tightly packed activated carbon column to remove AAA-containing peptides and recover the BCAA-rich peptides in the flow-through fraction. To improve the elution of the peptides from the column, the flaxseed protein hydrolysates were acidified prior to loading onto the activated carbon column. This treatment was necessary to precipitate any large peptides and residual undigested proteins, which have high adsorption capacity on activated carbon (*14*) and may block the activated carbon matrix thereby decreasing elution efficiency. The absorbance values of the flow-through sample at 280 and 260 nm, compared to the crude hydrolysate solution, showed that over 90% of AAA were removed from the FPH. However, data from amino acid analysis of the freeze-dried powder showed that the activated carbon column specifically removed only 39−57% of AAA from FPH. This resulted in an overall increase in the Fischer ratio of the resulting FPH-col peptide samples when compared with the crude FPH (**Table 1**). However, the Fischer ratios of the FPH-col flow-through samples were low, ranging from 2.5 to 4.04 representing up to 2.3-fold increase relative to the crude FPH. The highest Fischer ratio (4.04) was observed for the

Table 2. Percentage Amino Acid Composition of Flaxseed Protein Isolate (FPI), Thermolysin−Pronase Hydrolysate (FPH), FPH Passed through a Column Packed with Activated Carbon (FPH-col) and FPH Mixed with Activated Carbon inside a Centrifuge Tube (FPH-mix)

amino acids	FPI	FPH	FPH-col	FPH-mix
Asx[a]	11.29	10.83	11.40	8.27
Thr	3.67	3.60	4.40	4.36
Ser	4.34	4.99	6.28	5.79
Glx[b]	19.83	21.30	17.79	13.65
Pro	5.29	4.08	4.12	2.57
Gly	5.47	5.22	5.50	4.48
Ala	4.89	4.47	6.70	6.80
Cys	1.36	1.38	0.58	0.18
Val	5.20	5.72	5.71	10.10
Met	2.02	1.49	1.10	0.76
Ile	4.50	5.14	4.35	7.56
Leu	5.80	5.93	6.61	11.34
Tyr	2.52	2.78	1.19	0.28
Phe	5.47	5.52	2.36	0.83
His	2.29	2.23	3.12	3.27
Lys	3.04	3.03	4.98	5.97
Arg	11.28	10.79	13.23	13.68
Trp	1.73	1.48	0.57	0.12
AAA[c]	9.73	9.78	4.13	1.23
BCAA[d]	15.51	16.79	16.68	29.00
Fischer ratio	1.59	1.72	4.04	23.65
Phe + Tyr	7.99	8.30	3.55	1.11

[a] Asx, aspartic acid + asparagine. [b] Glx, glutamic acid + glutamine. [c] AAA: phenylalanine, tyrosine, tryptophan. [d] BCAA: valine, leucine, isoleucine.

hydrolysate product of thermolysin−pronase activity. It could be observed that increase in the Fischer ratio correlated with increase in the cleavage sites of the enzymes, except for alcalase. This showed that the less specific enzymes liberated smaller peptides that passed through the packed activated carbon in the column. The low Fischer ratio obtained in the flow-through samples indicated that some of the BCAA-containing peptides may have been trapped in the activated carbon matrix in the column. The result obtained in this study is contrary to that of a previous study that reported the preparation of protein hydrolysates with high Fischer ratio of 31.6 by enzymatic hydrolysis of casein and passage of the resulting acidified hydrolysates through an activated carbon-packed column (*14*). Recently, Ma et al. (*11*) also used similar activated carbon treatment to prepare a peptide mixture with Fischer ratio of 34.71 from Alcalase−papain hydrolyzed corn gluten meal. The differences in our results when compared with previous reports may be due to differences in the nature of the substrate, i.e., flaxseed proteins compared to casein and corn gluten meal. Due to the low Fischer ratio of the FPH-col, another approach was adopted to minimize BCAA loss while effectively removing the AAA-containing peptides and free AAA.

Vortexing of the FPH with activated carbon in a plastic centrifuge tube resulted in concentration-dependent removal of AAA and decrease in the protein content. The optimized condition (2.5% activated carbon, w/v) that gave the highest peptide yield (unadsorbed peptides as A_{220nm}) with the removal of most of the AAA was used for the scale-up experiment. Data from amino acid analysis of the freeze-dried filtrate showed that the activated carbon resulted in the removal of 33−87% of AAA. The activated carbon treated FPH-mix sample from thermolysin−pronase activity had the highest amount of BCAA (29%) and lowest amount of phenylalanine + tyrosine (1.11%) with a Fischer ratio of 23.65, representing 13.7-fold increase relative to the crude FPH and 14.8-fold increase relative to the intact flaxseed protein (**Table 2**). In this sample, vortexing decreased the AAA composition from 9.78% in the crude FPH to 1.23%

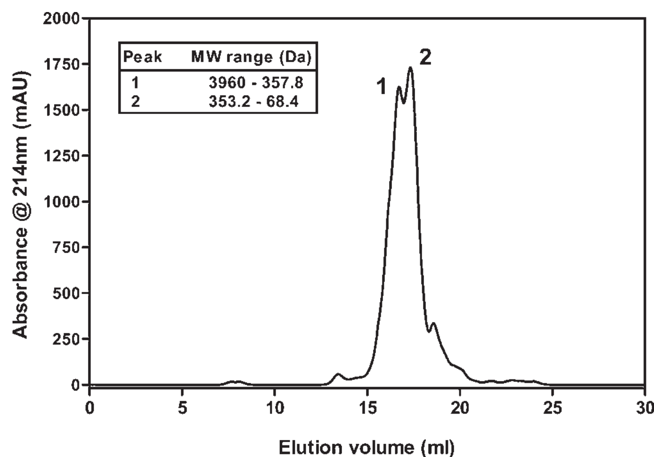

Figure 2. Size-exclusion gel chromatogram of the high Fischer ratio FPH-mix.

with up to 2-fold enrichment of BCAA (**Table 2**). After similar activated carbon treatment, the other FPH-mix samples from papain, ficin and alcalase treatments had lower Fischer ratio of 2.8−6.2 due to their high AAA contents, although there was an increase in the Fischer ratio when compared with the crude FPH and isolated flaxseed protein. Protein hydrolysates or peptide mixtures of Fischer ratio higher than 20 and phenylalanine + tyrosine value lower than 2% can be used to treat patients with liver diseases (*7*); thus, these results show that the highest Fischer ratio flaxseed peptide mixture from thermolysin−pronase treatment has promising application for use in treating liver diseases in humans. Other recent studies have reported the production of peptide mixtures with Fischer ratios 24.58, 28.3, and 30.6 respectively from pearl oyster meat protein (*10*), *Brassica carinata* protein (*12*) and bovine casein (*13*) generated through enzymatic hydrolysis, activated carbon treatment and gel filtration chromatography. Thus, the process reported in this paper represents a cost-effective method of production of high Fischer ratio peptide mixture from flaxseed protein since activated carbon is cheap and can be regenerated, and our process did not require the expensive gel filtration chromatography protocol.

Estimated Peptide MW Distribution. The MW of peptides is important in determining their absorption following oral intake. It is believed that low-MW peptides can be efficiently transported across the enterocytes through several peptide transporters. MW estimation using gel permeation chromatography showed that the high Fischer ratio FPH-mix contained peptides of sizes < 3.96 kDa (**Figure 2**). The first peak (1) contains oligopeptides of MW 357−3960 Da whereas peak 2 contains mostly dipeptides, tripeptides and free amino acids as indicated by the MW range of 68−353 Da. The low-MW peptide content of this sample increases its potential for use as nutraceutical in treating liver diseases.

Antioxidant Activity. DPPH radical is an N-containing radical used for the primary screening of antioxidant capacity of food components. The high Fischer ratio FPH-mix showed moderate DPPH radical scavenging activity at 5 mg protein/mL compared to glutathione (**Figure 3**); dose-dependent assay was not conducted due to the low activity of the peptide sample in scavenging DPPH radical. This activity is weak compared to the DPPH radical scavenging activity previously reported for 1 mg/mL flaxseed protein hydrolysates (*19*) and 1.6 mg/mL alfalfa leaf protein hydrolysates (*31*), which scavenged more than 60% of DPPH radical. However, the high Fischer ratio FPH-mix showed concentration-dependent scavenging of $O_2^{\bullet -}$ produced from pyrogallol autoxidation (**Figure 4**) with a 50% effective concentration (EC_{50}) value of 1.67 ± 0.19 mg protein/mL. At 4 mg

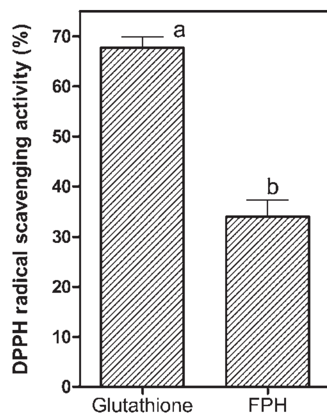

Figure 3. DPPH radical scavenging activity of 5 mg protein/mL of the high Fischer ratio FPH-mix compared to glutathione.

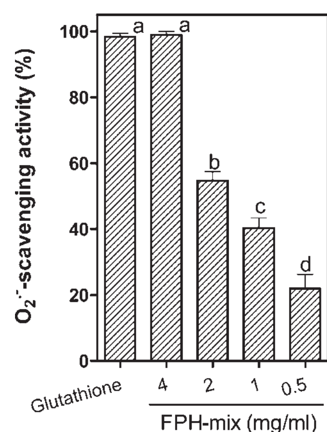

Figure 4. Concentration-dependent scavenging of superoxide radical ($O_2^{\bullet -}$) by high Fischer ratio FPH-mix with effective concentration (EC_{50}) value of 1.67 mg protein/mL; bars with different letters are significantly different at $p < 0.05$.

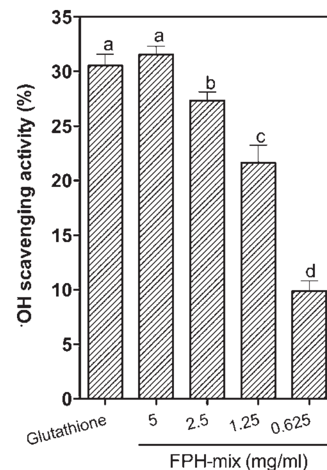

Figure 5. Concentration-dependent scavenging of hydroxyl radical (•OH) by BCAA-rich FPH-mix; bars with different letters are significantly different at $p < 0.05$.

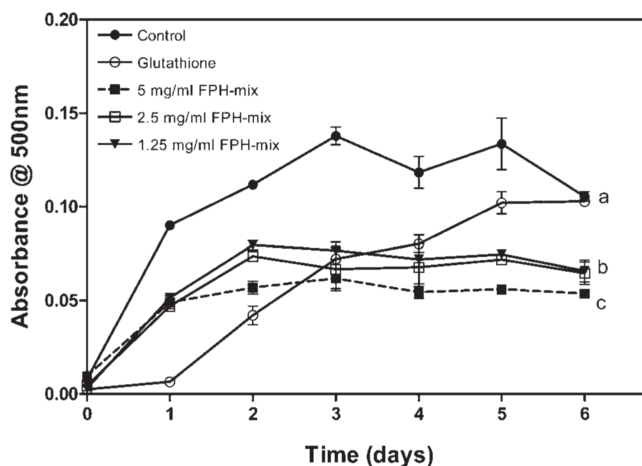

Figure 6. Protective effect of different concentrations of the high Fischer ratio FPH-mix and glutathione against linoleic acid oxidation.

protein/mL, the sample scavenged almost 100% of the $O_2^{\bullet -}$ produced in the reaction. In comparison, this antioxidant activity is stronger than the $O_2^{\bullet -}$ scavenging of low-MW flaxseed protein-derived peptides (*19*), but similar to the activity of three chickpea protein hydrolysate fractions that scavenged 47−69% of $O_2^{\bullet -}$ at 2 mg/mL (*29*). Excessive $O_2^{\bullet -}$ produced during oxidative stress, from mitochondrial electron leakage and activated macrophages, is implicated in the oxidative damage of biological macromolecules associated with degenerative diseases in humans. Thus, the high Fischer ratio peptide mixture produced in this work could offer protection to cellular components against these oxidative damages.

In addition, the flaxseed peptide mixture displayed moderate scavenging of •OH in a concentration-dependent fashion with a maximum scavenging of 31.5% at 5 mg protein/mL whereas glutathione showed similar scavenging activity at a lower concentration of 1 mg/mL (**Figure 5**). This activity is weaker than those previously reported for low-MW peptides from flaxseed protein that scavenged more than 70% of •OH at <1.5 mg/mL (*19*) and chickpea protein hydrolysate fractions, which scavenged 38−81% of •OH at 1.5 mg/mL (*29*). The moderate •OH scavenging activity of FPH-mix may be due to the low levels of phenylalanine present in the peptide mixture. This is because phenylalanine possesses strong radical-scavenging property since it can trap •OH via hydroxylation of the aromatic ring leading to the formation of stable *o*-, *m*- or *p*-tyrosine (*32*).

The FPH-mix with highest Fischer ratio (BCAA-rich) was found to show strong inhibition of linoleic acid oxidation in an *in vitro* evaluation system. As observed in **Figure 6**, the increase in A_{500nm} indicates an increase in linoleic acid oxidation, and this occurred during the first three days followed by a gradual decrease during the next three days. On day 1, the BCAA-rich mixture (1.25−5 mg/mL) inhibited approximately 45% of linoleic acid oxidation whereas glutathione (1.25 mg/mL) was over 2-fold more active. As glutathione lost its activity in protecting linoleic acid from oxidation between days 2 and 6, the BCAA-rich sample maintained antioxidant property at the three concentrations. Glutathione lost its activity with time since its antioxidant function group, the −SH group of cysteine, could not have been regenerated during the assay. In the last two days of the experiment, 5 mg/mL of the BCAA-rich sample displayed the best inhibitory property against linoleic acid oxidation ($P < 0.05$). On the sixth day, 5, 2.5, and 1.25 mg/mL of the peptide mixture concentration-dependently inhibited linoleic acid oxidation by 48, 37.5 and 36.4%, respectively, whereas glutathione completely lost its activity. In contrast, a chickpea protein hydrolysate fraction displayed a stronger activity by inhibiting *in vitro* linoleic acid peroxidation by 81% at 1 mg/mL after 8 days of incubation (*29*). The oxidation of polyunsaturated fatty acid components of biological membranes is an important mechanism of

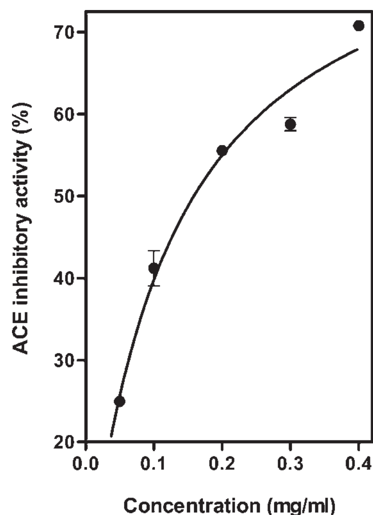

Figure 7. Concentration-dependent inhibition of ACE activity by high Fischer ratio FPH-mix with 50% inhibitory concentration (IC_{50}) value of 0.16 mg protein/mL.

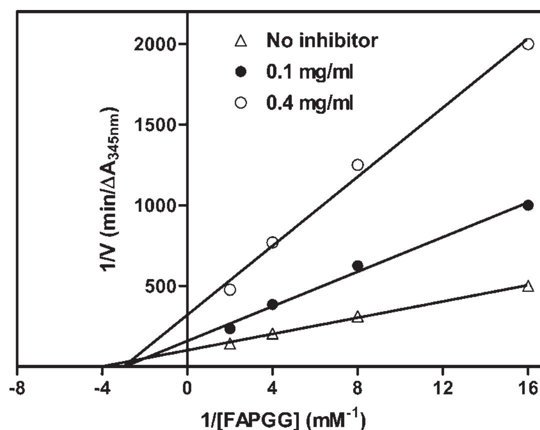

Figure 8. Double reciprocal plot of the inhibition of ACE activity by 0.1 and 0.4 mg/mL of the high Fischer ratio FPH-mix indicating noncompetitive mode of inhibition.

cellular damages induced by reactive oxygen species; thus, the BCAA-rich sample from flaxseed protein may offer protection to cellular components by reducing oxidative damages.

Potential Antihypertensive Properties. In addition to its antioxidant properties, the BCAA-rich sample also displayed potential antihypertensive property as evident from the concentration-dependent inhibition of ACE activity with up to 70.8% ACE inhibition at 0.4 mg/mL and 50% inhibitory concentration (IC_{50}) value of 0.16 ± 0.0055 mg/mL (**Figure 7**). This IC_{50} value is comparable to the ACE-inhibitory activity of low-MW FPH from pancreatin hydrolysis (IC_{50} = 0.151 mg/mL), but weaker than the activity observed for <1 kDa thermolysin prepared FPH with IC_{50} value of 0.0275 mg/mL (*20*) and crude α-zein hydrolysates with IC_{50} value of 0.021 mg/mL (*33*). Considering the role of hydrophobic amino acids in ACE inhibition (*34*), it could be that the decreased amount of AAA in the BCAA-rich sample contributed to the decreased potency of the peptides in ACE inhibition when compared to the previously reported flaxseed peptide mixtures (*20*).

Kinetic studies of ACE inhibition using the Lineweaver−Burk plot showed that the BCAA-rich sample inhibited ACE in a mixed-type inhibition pattern (**Figure 8**). This was characterized by an increase in K_m from 0.3121 mM for the uninhibited reaction to 0.6179 and 0.5860 mM in the presence of 0.1 and 0.4 mg/mL peptides, respectively. The reaction V_{max} also decreased in a concentration-dependent fashion from 0.0112 Δ*A*/min for the uninhibited reaction to 0.009416 and 0.004521 Δ*A*/min in the presence of 0.1 and 0.4 mg/mL of the inhibitor, respectively. These results are similar to the mode of ACE inhibition reported for low-MW crude and cationic peptide fractions from flaxseed protein (*20*). This indicates that the constituent peptides in the BCAA-rich sample inhibited ACE activity by binding both the enzyme active and allosteric sites; in other words, the peptides (or amino acids) interacted with ACE in both its free and substrate-bound forms. This shows that ACE inhibition by the BCAA-rich sample cannot be completely overcome by increasing the substrate concentration. The catalytic efficiency of ACE was observed to decrease from 0.0358 to 0.0152 and 0.0077 in the presence of 0.1 and 0.4 mg/mL of the peptides, respectively, which indicates that inhibition of ACE activity by the BCAA-rich sample was concentration-dependent. In contrast to these results, Cinq-Mars et al. (*35*) reported that both fractionated and unfractionated Pacific hake fillet hydrolysates exhibited competitive mode of ACE inhibition using hippuryl-histidyl-leucine as ACE substrate. Information on the kinetics of ACE inhibition by a peptide mixture could contribute to the elucidation of its mode of action prior to *in vivo* blood pressure-lowering studies.

In addition to ACE inhibition, molecules that can inhibit the activity of renin can potentially provide better blood pressure lowering activity since renin controls the rate-limiting step of the renin−angiotensin system. In our present study, the BCAA-rich sample did not show any considerable inhibitory activity against renin; the peptide mixture inhibited 7.1% renin activity at 4 mg/mL. In contrast, a recent study reported that low-MW flaxseed protein-derived peptides moderately inhibited human renin *in vitro* by 50% at concentrations ranging from 1.21 to 2.81 mg/mL (*20*). The renin−angiotensin system has been widely studied as target for the treatment of hypertension in humans, and a wide range of food proteins have afforded potent ACE-inhibiting peptides, some with antihypertensive activities in animals and humans.

In conclusion, treatment of flaxseed protein isolate with thermolysin and pronase followed by simple mixing with activated carbon afforded a BCAA-rich mixture with high Fischer ratio. The multifunctional bioactive properties of the peptide will potentially enhance the value-added utilization of defatted flaxseed meal by making it an important raw material in the functional foods and nutraceuticals industry. Animal feeding experiments using this BCAA-rich sample preparation need to be conducted in animal model of liver disease to evaluate the *in vivo* effects of the peptides in improving plasma Fischer ratio and reducing muscle wasting.

ABBREVIATIONS USED

AAA, aromatic amino acids; ACE, angiotensin I-converting enzyme; BCAA, branched-chain amino acids; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FAPGG, *N*-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine; FPI, flaxseed protein isolate; FPH, flaxseed protein hydrolysates; LA, linoleic acid; MW, molecular weight.

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