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Release of Angiotensin I-Converting Enzyme Inhibitory Peptides from Flaxseed (*Linum usitatissimum* L.) Protein under Simulated Gastrointestinal Digestion

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ABSTRACT: The scope of this study was to determine the ability of flaxseed (*Linum usitatissimum* L.) proteins to release angiotensin I-converting enzyme inhibitory (ACEI) peptides during simulated gastrointestinal (GI) digestion using a static (SM; no absorption in the intestinal phase) and a dynamic model (DM; simultaneous absorption of digested products in the intestinal phase via passive diffusion). Gastric and gastric + small intestinal digests of flaxseed proteins of both models possessed ACEI activity. The ACEI activity of the gastric + small intestinal digest in the DM (IC_{50} unabsorbed, 0.05 mg N/mL; IC_{50} absorbed, 0.04 mg N/mL) was significantly higher ($p < 0.05$) than that of the SM (IC_{50}, 0.39 mg N/mL). Two peptides, a pentapeptide (Trp-Asn-Ile/Leu-Asn-Ala) and a hexapeptide (Asn-Ile/Leu-Asp-Thr-Asp-Ile/Leu), were identified in the most active ACEI fraction (0.5−1 kDa) of the absorbable flaxseed protein digest by de novo sequencing.

KEYWORDS: Flaxseed, *Linum usitatissimum* L., angiotensin I-converting enzyme, peptide, simulated gastrointestinal digestion, static model, dynamic model, retentate, dialysate, de novo sequencing

INTRODUCTION

At present, consumer attention to health benefits of food has tremendously increased with the emergence of the functional food phenomenon. Flaxseed (*Linum usitatissimum* L.) is one of the major oilseeds studied today as a functional food besides soy. The seed is a valuable source of oil (30−40%), protein (20−25%), and dietary fiber (20−28%).[1] Reported health benefits of flaxseed include reduction of atherogenic risk; anticancer, antiviral, bactericidal, and anti-inflammatory activities; and laxative effects.[2] The consumption of whole flaxseed and its meal as well as flaxseed constituents has demonstrated protection against cardiovascular disease (CVD).[3] Studies to identify the cardioprotective components in flaxseed have focused on the α-linolenic acid (ALA) of the oil, lignan, and mucilage.[4,5] Findings of these studies are inconsistent, and the specific cardioprotective component of flaxseed has not been completely elucidated. The antiatherogenic effect in defatted flaxseed meal and absence of any atherogenic effect by lignans, as shown by some studies,[3,6] suggest that a nonlipid and nonlignan component in flaxseed meal could be the contributory factor for protection against CVD. Flaxseed proteins have received very limited attention as a potential component with significant biological activity. Currently, the official Web sites of flax organizations[7,8] report cardioprotective benefits of flaxseed oil, lignans, and fiber but not of proteins, showing the absence of published scientific evidence on flaxseed protein and cardiovascular health.

Peptides with angiotensin I-converting enzyme inhibitory (ACEI) activities are key molecules to reduce elevated blood pressure,[9] which in turn provide cardiovascular health. The release of ACEI peptides has been reported from several seed storage proteins upon enzymatic digestion.[10−12] However, the in vitro studies that generated ACEI peptides under simulated gastrointestinal (GI) digestion lack the simulation of absorption/removal of digestion products in the intestinal phase. Therefore, ACEI peptides generated in these models may not represent the actual generation of bioactive peptides under physiological conditions. Moreover, such in vitro models do not show the potential of ACEI peptides for absorption via the intestinal epithelium. An in vitro digestion model with continuous removal of digestion products during the small intestinal phase will represent a more realistic model that represents the in vivo digestion process.

The presence of ACEI activity in enzymatically hydrolyzed flaxseed protein in vitro has been reported by previous studies[14−17] in which the enzymes involved are either microbial proteases or individual proteolytic enzymes of the digestive tract. To the best of our knowledge, there is no published information available on the ability of flaxseed protein to generate ACEI peptides during GI digestion either in vitro or in vivo. Also, flaxseed protein derived ACEI peptides have not been sequenced. The objective of the present in vitro study was to investigate whether flaxseed protein generates ACEI peptides with the potential for absorption during simulated GI digestion. Flaxseed protein was subjected to simulated GI digestion using a static model (SM; no absorption of digested products) and a dynamic model (DM; simulated absorption via passive diffusion) for comparison purposes.

MATERIALS AND METHODS

Materials. The flaxseed variety Valour was used throughout the study. Pepsin (from porcine gastric mucosa, 2490 units/mg solid; 3410 units/mg protein), pancreatin (from porcine pancreas, 8 × USP), ACE

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(from porcine kidney; ≥10 units/mg protein), and other chemicals used were purchased from Sigma Chemical Co. (Sigma-Aldrich Canada Ltd., Oakville, Canada). The Megazyme total dietary fiber analysis kit was from Megazyme International Ireland Limited (Ireland). All of the solvents used for chromatography were of high-performance liquid chromatography (HPLC) grade, and reagents were of analytical grade.

Extraction of Flaxseed Proteins. Protein was extracted from demucilaged and defatted flaxseed meal using alkali solubilization (pH 8.5) followed by acid precipitation (pH 3.8) as previously described in Marambe et al.[14]

Analysis of Composition. *Proximate Composition.* The analysis of moisture, ash, crude protein, and crude fat was carried out using AOAC[18] procedures. The total dietary fiber (TDF), insoluble dietary fiber (IDF), and soluble dietary fiber (SDF) of samples were analyzed using a Megazyme total dietary fiber analysis kit.

Amino Acid Analysis. Samples were oxidized with performic acid, hydrolyzed with HCl,[19] and analyzed using an AMINOSep ion exchange column (particle size, 5 μM; capacity, 2 mequiv/g; Transgenomics Inc., United States) connected to a HPLC system (Agilent 1100 series, Agilent Technologies, Germany) and fluorescence detector (SHIMADZU, Japan) set at Ex 233 nm and Em 351 nm. Post column *o*-pthalaldehyde (OPA) derivatization was used for the identification of amino acids. For the analysis of Trp, samples were predigested in 5 mL of 5 N NaOH with 100 mg of potato starch for 5 h at room temperature followed by incubation at 110 °C for 24 h. After the pH was adjusted to between 2.5 and 3.5 with 6 N HCl, the amino acid analysis was carried out using an analytical reversed phase column (250 mm × 4.6 mm, 5 μ, Luna, Phenomenex, Canada) connected to the above HPLC system. The method was validated for precision, accuracy, reproducibility, and analyte recovery. Calibration was performed using tryptophan standard, with the correlation R^2 values for calibration curves using five or more data points routinely being better than 0.999. The reproducibility tests based on duplicate analysis of wide range of samples with various matrices showed mean coefficients of variation less than 5%, and the recovery of analyte from samples spiked with the authentic standard ranged from 95 to 100%.

Simulated GI Digestion of Flaxseed Protein. *Preparation of Digestive Fluids.* The simulated digestive fluids were prepared fresh daily as described in the United States Pharmacopeia[20] with some modifications as follows.

Simulated Gastric Control Solution (SGFc) and Simulated Gastric Fluid (SGF). The SGFc (pH 2.0) contained 0.2% (w/v) NaCl in deionized water. The SGF was prepared by mixing pepsin with the SGFc (stored at 4 °C) to achieve a concentration of 4 mg/mL.

Simulated Intestinal Control Solution (SIFc) and Simulated Intestinal Fluid (SIF). The SIFc (pH 6.8) had a composition of 0.68% (w/v) KH_2PO_4 and 0.062% (w/v) NaOH in deionized water. The SIF was prepared to have 10 mg/mL pancreatin in SIFc.

Simulated Bile Solution. The simulated bile solution consisted of sodium taurocholate and sodium glycodeoxycholate at 4 mM each in SIFc prepared as discussed above.

Simulated GI Digestion. *Simulated Gastric Digestion.* Flaxseed protein (1 g) was incubated with SGFc (25 mL) in a 50 mL Erlenmeyer flask for 10 min at 37 °C in a shaking water bath (GRANT instruments, Hillsborough, NJ). The solution was brought to pH 2.0 ± 0.1 with 1 M HCl, and the total volume was made up to 29 mL with SGFc. Freshly prepared SGF (1 mL) was added into this solution to result in a pepsin/protein ratio (E/S) of 1:250 w/w, and the mixture was subsequently incubated in a shaking water bath (167 rpm) at 37 °C for 2 h. An enzyme blank was prepared by incubating the enzyme under the conditions described with the substrate omitted. A sample blank contained sample and the SGFc and was incubated using the same conditions. The gastric digestion phase was terminated by inactivating pepsin by raising the pH of the solution to 6.8 with the addition of 1 M NaOH.

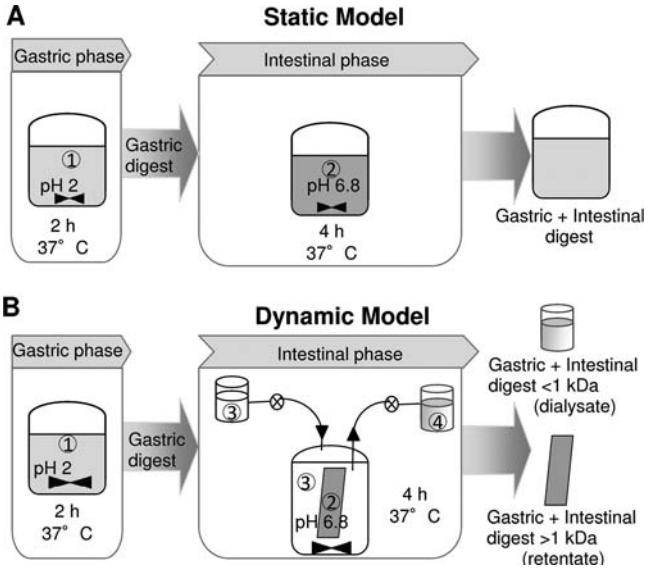

Figure 1. Schematic diagram of (A) SM and (B) DM used for flaxseed protein digestion. 1, Sample in SGF containing pepsin; 2, gastric phase digest in SIF containing pancreatin and bile; 3, SIFc; and 4, SIFc containing <1 kDa molecules.

Simulated Intestinal Digestion. SM. The gastric phase digested sample (pH 6.8) was adjusted to 35 mL by adding SIFc and incubated for 5 min at 37 °C in a shaking water bath. To this mixture, 1 mL of bile solution and 4 mL of SIF (previously maintained at 37 °C) containing pancreatin (E/S; 1:25 w/w) were added and incubated (167 rpm) for 4 h at 37 °C while mixing (Figure 1A). The same protocol was also carried out for sample blanks and enzyme blanks as described under gastric phase digestion.

DM. The DM (Figure 1B) used in the present study was a simplified model of the two-step proteolysis model developed by Savoie and Gauthier.[21] In this model, a dialysis bag made of Spectra/Por dialysis membrane (flat width, 45 mm; diameter, 29 mm) with a molecular weight cut off (MWCO) value of 1 kDa was used as the simulated small intestinal compartment. The gastric digest (pH 6.8) volume was adjusted to 35 mL using SIFc and transferred to the dialysis bag. One milliliter of bile solution and 4 mL of SIF containing pancreatin (enzyme: substrate 1:25 w/w), previously maintained at 37 °C, were added to the dialysis bag, and digestion was continued for 4 h with continuous stirring. In this setup, the dialysis bag was immersed in buffer (similar in composition to SIFc, pH 6.8, 1000 mL) containing vessel maintained at 37 °C while mixing. This vessel was connected to a buffer feeding reservoir (at 37 °C) and a receiving flask. The buffer in which the dialysis bag was immersed was constantly replenished from the feeding reservoir at a transfer rate of 1.6 mL/min using a peristaltic pump (Figure 1B). Buffer with permeated digestion products was transferred to the receiving flask at the same transfer rate. Buffer collected in the receiving flask and also in the dialysis membrane containing vessel was collected at the end of intestinal phase digestion. A sample blank (no enzymes added) and an enzyme blank (no sample added) that has gone through gastric phase digestion were also subjected to intestinal phase digestion in similar manner.

Collection of Samples. Samples were collected at zero time of digestion (undigested protein), at the end of gastric phase (gastric digest) and gastric + small intestinal phase (gastric + small intestinal digest). In the DM, samples of dialysate and retentate of the gastric + small intestinal digest were collected separately at the end of digestion. Collected samples were heated at 98 °C for 15 min to inactivate the enzymes, cooled to room temperature, adjusted to pH 3.8 (pH at which

flaxseed protein shows minimum solubility, based on preliminary studies), and centrifuged at 13800*g* for 15 min. The volume of the recovered supernatant (digest) was recorded, and the pH was adjusted to 7 using 1 M NaOH. The resulting solution was adjusted to a known volume using deionized water in a volumetric flask. Aliquots of this solution were used for the determination of the degree of hydrolysis (DH) and ACEI activity.

Measurement of DH and Average Peptide Chain Length (PCL). The DH, which is defined as the percentage of peptide bonds cleaved, was calculated based on the free amino groups of the digested products determined according to the OPA method as described by Wanasundara et al.[22] The total number of amino groups in the flaxseed protein was estimated using acid hydrolysis (6 N HCl at 110 °C for 24 h). The average PCL was calculated from DH according to Adler-Nissen.[23]

Determination of ACEI Activity. The ACEI activity of the flaxseed protein and digests (freeze-dried) was assessed using Hippuryl-His-Leu as the substrate. The assay for ACE activity and released hippuric acid due to uninhibited ACE activity was determined as described by Wanasundara et al.[24] with modifications in the mobile phase (25%, v/v acetonitrile in deionized water at pH 3.0 adjusted by adding glacial acetic acid) and detection wavelength (238 nm). The control assay mixture had ACE buffer instead of the digest. The percent inhibition of ACE activity was calculated as described by Wanasundara et al.[24] The IC_{50} value for ACE inhibition (the concentration of digest required to inhibit 50% of the ACE activity) was calculated by using different concentrations of digests.

Determination of Molecular Mass Distribution Profile of Flaxseed Protein Digests. Samples (freeze-dried; 20 mg/mL) were dissolved in the mobile phase (20% v/v acetonitrile + 0.05% v/v trifluoroacetic acid/TFA), filtered through a 0.45 μm membrane, and injected (150 μL) manually on to a Superdex peptide HR 10/30 size exclusion column (SEC) (column size 10 mm × 300 mm, Amersham Pharmacia Biotech, Sweden) connected to an AKTA Explorer FPLC system (Amersham Pharmacia Biotech). Elution was allowed under isocratic conditions (20% v/v acetonitrile + 0.05% v/v TFA) at a flow rate of 0.5 mL/min at room temperature. The column eluant was monitored at 214 and 280 nm. Standards of known molecular masses (insulin chain A, angiotensin II, and Hippuryl-His-Leu) were also separated on the same column to estimate the distribution of molecular masses of the peptides in the digests.

Peptide Fractionation. The dialysate of gastric + small intestinal digest of the DM was further fractionated into peptides based on the molecular masses of standards used. Fractions of eluant (1 mL) were collected using a fraction collector (Amersham Pharmacia Biotech), pooled based on the molecular mass distribution, and then freeze-dried. The freeze-dried fraction was assessed for ACEI activity after redissolving in buffer used for ACE assay.

De Novo Sequencing of Peptides. The lyophilized FPLC fraction of the dialysate from the DM with high ACEI activity was further purified using PepClean C-18 spin columns (Pierce, United States) according to manufacturer instructions. The amino acid sequences of the most abundant peptides in the fraction were determined using an ultra performance liquid chromatography system (UPLC; Waters, United Kingdom) connected to a high definition mass spectrometer (MS) (Synapt HDMS, Waters) interfaced with electrospray ionization (ESI) source, which was operated in the positive ion mode at a capillary voltage of 3.5 V. The samples were separated on a 2.1 mm × 50 mm Acquity reverse phase column (BEH, C18, 1.7 μm) at 65 °C, using 1% (v/v) formic acid (solvent A) and acetonitrile (solvent B) as the mobile phases. A gradient elution was employed starting at 10% (v/v) B and rising linearly to 90% (v/v) within 15 min. The composition was returned to initial conditions within 2 min and thereafter to 5% (v/v) B within another 2 min giving a total run time of 19 min. The injection volumes of the samples were 10 μL. Flow (0.6 mL/min) from the UPLC column entered into the MS. The cone voltage and collision energy for peptides were 40 V and 30 eV, respectively, whereas the ion source and desolvation temperature were at 100 and 150 °C, respectively. The MS/MS spectra were obtained for the most abundant peptides in the total ion current chromatogram (TIC). The amino acid sequencing of the peptides was performed using Masslynx/MASCOT distiller programs (Micromass, United Kingdom) as de novo sequencing.

Statistical Analysis. All of the experiments and analyses were conducted at least in duplicate. All of the values were reported as means ± standard deviations. The results were evaluated using a generalized linear model [one-way analysis of variance (ANOVA)] with multiple comparisons of means (Tukey's pair wise comparisons). Statistical analysis was performed using SAS 9.1 software (SAS Institute Inc., United States).

RESULTS

Composition of Extracted Flaxseed Protein. The extracted flaxseed protein had 86.01 ± 0.20% protein (N% × 6.25), 0.55 ± 0.08% fat, 8.01 ± 0.60% TDF (5.41 ± 0.75% SDF and 2.59 ± 1.35% IDF), 5.98 ± 0.94% moisture, and 4.13 ± 0.25% ash. The SDF, which comprised the majority of the TDF, was expected to be the soluble polysaccharides (mucilage) that was not removed during demucilaging and also those polysaccharides extracted from seed embryo and endosperm cell walls during protein recovery. The extracted protein was rich in acidic amino acids such as Glu and Asp as well as the basic amino acid, Arg (Table 1). The other amino acids found in abundance were the neutral, hydrophobic amino acids, Gly and Leu. The amino acids His, Trp, Tyr, S-containing amino acids (Cys and Met), Lys, and Pro were found in low concentrations in flaxseed protein. The ratio between essential and total amino acids (E/T %) of flaxseed protein was 34.29%, and the Lys:Arg ratio was 0.22.

The DH and Average PCL of Digested Flaxseed Protein. The undigested flaxseed protein showed a low DH of 3.31 ± 0.03% (PCL of 30.2), which increased progressively with gastric and small intestinal phases of digestion. The gastric digestion resulted in a DH of 7.96 ± 0.16% (~13 PCL), while the gastric + small intestinal digests obtained from both models had significantly higher ($p < 0.05$) DH than that of the gastric digest (Table 2). The DH of gastric + small intestinal digest of the DM (46.78 ± 0.3%) was significantly higher ($p < 0.05$) than that of the SM (DH, 43.95 ± 0.8%). However, the average PCL in both digests was found to be ~2.

ACEI Activity of Digested Flaxseed Protein. The digests of flaxseed protein were screened for ACEI activity prior to determining IC_{50} values (Table 3). The gastric and gastric + small intestinal digests of flaxseed protein obtained from both SM and DM possessed ACEI activity. The undigested flaxseed protein did not show any ACEI activity. Therefore, the IC_{50} for ACEI activity was not determined for the undigested flaxseed protein. The gastric phase digest had an ACEI activity of 81.32 ± 1.93% at the concentration employed with an IC_{50} of 0.16 ± 0.00 mg of N/mL (Table 3), indicating the release of ACEI peptides from flaxseed protein by the action of pepsin.

The ACEI activity of gastric + small intestinal digest of the SM (52.01 ± 1.89%; IC_{50}, 0.39 ± 0.00 mg of N/mL) was significantly lower ($p < 0.05$) as compared to that of the gastric digest (Table 3). The freeze-dried dialysate of the gastric + small intestinal digest obtained from the DM consisted of low N levels of 0.77 ± 0.01% as compared to the retentate (7.96 ± 0.03%). Therefore, to screen for ACEI activity, a higher concentration of dialysate was used (6.67 mg/mL) than the retentate (0.67 mg/mL) to overcome any errors associated with the dilution effect of

Table 1. Total Amino Acid Composition of Isolated Flaxseed Protein

amino acid	g/100 g protein[a]	amino acid requirement for adults (mg/kg/day)[b]
	essential amino acids	
His	1.59 ± 0.02	8−12
Ile[c]	3.37 ± 0.01	10
Leu[c]	4.67 ± 0.03	14
Lys	2.12 ± 0.04	12
Met + Cys	3.74 ± 0.14	13
Phe + Tyr	6.02 ± 0.01	14
Thr	2.83 ± 0.19	7
Trp	1.10 ± 0.02	3.5
Val[c]	3.75 ± 0.03	10
	nonessential amino acids	
Ala	3.77 ± 0.16	
Arg	9.71 ± 0.00	
Asp	9.02 ± 0.08	
Glu	19.75 ± 0.36	
Gly	5.19 ± 0.08	
Pro	2.45 ± 0.07	
Ser	4.53 ± 0.07	
Lys/Arg	0.22	
E/T (%)[d]	34.29	

[a] Values are means ± standard deviations. [b] From ref 25. [c] Branched chain amino acids. [d] E/T, ratio between essential amino acids and total amino acids.

peptides. At the concentrations of dialysate and retentate used, both showed ACE inhibition (dialysate, 52.96 ± 1.81%; and retentate, 59.12 ± 2.03%). However, there was no significant difference between these two for IC_{50} values (dialysate, 0.04 ± 0.00 mg N/mL; and retentate, 0.05 ± 0.00 mg N/mL). The gastric + small intestinal digest of the DM had significantly higher ($p < 0.05$) ACEI activity than that of the gastric digest, which was in contrast to the findings of the SM. Interestingly, the gastric + small intestinal digest of the DM possessed significantly greater ($p < 0.05$) potential for ACE inhibition as compared to that of the SM as shown by the IC_{50} values (Table 3).

Amino Acid Profile of the Flaxseed Protein Digests. The amino acid composition of the digests of two models was compared separately. A difference of ≥0.1% between amino acid content was taken as a considerable difference. The percentage of His, Ile, Lys, Met + Cys, Val, and Gly were considerably higher (≥0.1%) in the gastric + small intestinal digest of the SM than the gastric digest (Table 4). The amino acid profile of the gastric + small intestinal digest of the SM was different than that of the DM. A major difference observed was the presence of the aromatic amino acid Tyr in the gastric + small intestinal digest of the DM, which was absent in the digest of the SM and in the gastric digest. The retentate of the gastric + small intestinal digest of the DM had a considerably higher percentage (≥0.1%) of ILe, Lys, Phe, Tyr, Thr, Val, Asp, Gly, Pro, and Ser than the gastric digest. The dialysate and retentate of the gastric + small intestinal digest (DM) also showed a considerable difference in terms of their amino acid profile. The dialysate of the DM had higher levels of Leu, Lys, Met, Phe, Tyr, Trp, Ala, Arg, and Ser than the retentate.

Table 2. DH and Average PCL of Flaxseed Protein Digests[a]

sample	DH (%)[b]	average PCL
gastric digest	7.96 ± 0.16 a	12.56 b
gastric + small intestinal digest		
SM	43.95 ± 0.80 b	2.30 a
DM	46.78 ± 0.31 c	2.13 a

[a] Means in the same column with different letters are significantly different ($p < 0.05$). [b] Values are means ± standard deviations.

Table 3. ACEI Activity of Flaxseed Protein Digests[a]

	ACEI activity	
sample	%[b]	IC_{50} (mg N/mL)[b]
initiation of digestion	NA	ND
gastric digest	81.32 ± 1.93	0.16 ± 0.00 b
gastric + small intestinal digest		
SM	52.01 ± 1.89	0.39 ± 0.00 c
DM		
retained fraction, >1 kDa	59.12 ± 2.03	0.05 ± 0.00 a
dialyzed fraction, <1 kDa	52.96 ± 1.81	0.04 ± 0.01 a

[a] Means in the same column with different letters are significantly different ($p < 0.05$). NA, no activity. ND, not determined. [b] Values are means ± standard deviations.

Molecular Mass Distribution Profile of Flaxseed Protein Digests. *Gastric Digest.* The gastric digest was comprised of peptides larger than 0.5 kDa (Figure 2). The presence of high molecular mass peptides as the majority in the gastric digest further confirmed the observed low DH (7.96 ± 0.16%) and long average PCL (12.5) values (Table 2). The relatively low absorbance at 280 nm indicated the presence of low levels of aromatic amino acids in the gastric digest.

Gastric + Small Intestinal Digest. The majority of the peptides in the gastric + small intestinal digest (SM) had molecular masses ranging from 2.5 to <0.5 kDa (Figure 3). There was a noticeable difference in the molecular mass distribution profile of both retentate and dialysate (Figure 4A,B) obtained from the DM. The retentate (Figure 4A) consisted of both large (>1 kDa) and small (<1 kDa) peptides with a majority of >1 kDa peptides. This shows that although the MWCO of the dialysis membrane was 1 kDa, there were notable amount of <1 kDa peptides from flaxseed protein digestion retained in the dialysis bag without diffusing out during the 4 h of small intestinal phase digestion. Both dialysate and retentate had peptides containing aromatic amino acids (Figure 4A,B, absorbance at 280 nm). Because the dialysate represented the bioavailable digestion products of flaxseed protein, it was fractionated into peptides of different molecular masses for detection of ACEI activity. Two fractions were collected from dialysate; GIF-I (0.5−1 kDa) and GIF-II (<0.5 kDa) (Figure 4B).

ACEI Activity of Peptide Fractions in the Flaxseed Protein Digests. Of the dialysate fractions, GIF-I (0.5−1 kDa) exhibited significantly higher ($p < 0.05$) ACEI activity (73.19 ± 0.75%) than GIF-II (<0.5 kDa; 1.36 ± 1.01%) (Table 5). The IC_{50} value for ACEI activity obtained for GIF-I was 3.71 ± 0.04 mg of solids/mL. The IC_{50} value was expressed as mg of solids/mL as the N% in the fraction was not determined due to limited sample recovery.

Table 4. Amino Acid Composition of Flaxseed Protein Digests

		gastric + small intestinal digest		
			DM	
amino acid (g/100 g protein)	gastric digest	SM	dialysate	retentate
		essential		
His	2.32	2.65	1.86	1.93
Ile[a]	4.00	4.22	3.93	4.46
Leu[a]	5.74	5.57	6.20	4.34
Lys	2.42	2.76	3.72	2.71
Met + Cys	1.62 + 2.16	1.64 + 2.42	1.86 + 0.62	1.29 + 1.59
Phe + Tyr	5.30 + 0.00	5.34 + 0.00	11.15 + 4.13	5.69 + 2.13
Thr	3.68	3.49	3.51	3.80
Trp	1.14	1.17	2.07	0.98
Val[a]	4.45	5.39	5.17	5.29
		nonessential		
Ala	4.53	4.43	4.55	4.24
Arg	11.36	11.38	18.8	10.57
Asp	11.74	11.13	8.88	13.79
Glu	23.78	22.72	13.43	20.9
Gly	6.03	6.81	4.75	6.27
Pro	2.77	2.28	2.69	4.14
Ser	5.66	5.28	5.99	5.85

[a] Branched chain amino acids.

De Novo Peptide Sequencing. The amino acid sequences and molecular masses of the abundant peptides in the most active ACEI gastric + small intestinal (absorbed) digest fraction (0.5−1 kDa) of DM were identified (Table 6). A pentapeptide (Trp-Asn-Ile/Leu-Asn-Ala) and a hexapeptide (Asn-Ile/Leu-Asp-Thr-Asp-Ile/Leu) were sequenced with more than 90% probability. Both peptides had Asp/Asn and Leu residues, which were abundant in isolated flaxseed protein (Table 1).

DISCUSSION

At present, there are no published studies on the generation of bioactive peptides during GI digestion of flaxseed protein. The current in vitro study showed that digestion of flaxseed protein under GI enzyme activity generated peptides that possess ACEI activity.

The amino acid profile of flaxseed protein used in the current study was similar to that reported by others.[26,27] According to Oomah and Mazza,[27] the amino acid pattern of flaxseed protein is comparable to that of soy, with both oilseeds having relatively high contents of Asp, Glu, Leu, and Arg. The results also suggested that flaxseed protein could be antiatherogenic and can contribute significantly to the supply of essential amino acids in the diet. The ratio of Lys to Arg of the extracted flaxseed protein (0.22) was lower as compared to that of proteins of Norman flaxseed (0.34)[26], soy and canola (0.88 for both).[28] L-Arg, which is the substrate of nitric oxide synthase (NOS), generates nitric oxide (NO) that acts as a vasodilator and an inhibitor of platelet aggregation.[29] Therefore, the low Lys:Arg is suggested to be the causal factor for antiatherogenic effect and antithrombotic effects of many plant proteins,[30] which indicates the higher potential of flaxseed protein to deliver antiatherogenic and antithrombotic effects than soy or canola proteins.

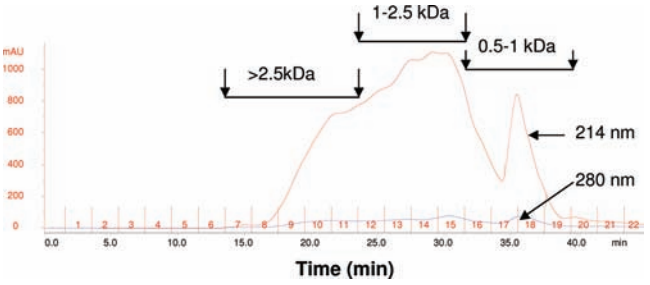

Figure 2. Molecular mass distribution profile of peptides in the gastric digest of flaxseed protein.

The choice of proteolytic enzymes in the present study was an important consideration because the specific action of enzymes on protein will influence the composition of digested products. The simulated GI digestion models included pancreatin as the intestinal phase enzyme instead of using trypsin and chymotrypsin. Pancreatin is a mixture of amylase, chymotrypsin, lipase, ribonuclease, and trypsin. Because food proteins occur mainly in close association with carbohydrates and lipids, it may be desirable to keep lipases and amylases in the enzymatic preparations. The activity of these nonproteolytic enzymes may influence the proteolytic enzymes, mainly by modifying their substrates.[31] Savoie and Charbonneau[32] have reported the importance of using pancreatin for in vitro digestion protocols as the pure enzymes such as trypsin and chymotrypsin are less stable in a mixture.

In the current study, flaxseed protein showed partial hydrolysis during the gastric phase. Enzymes employed in the small intestinal phase contributed more to flaxseed protein digestion

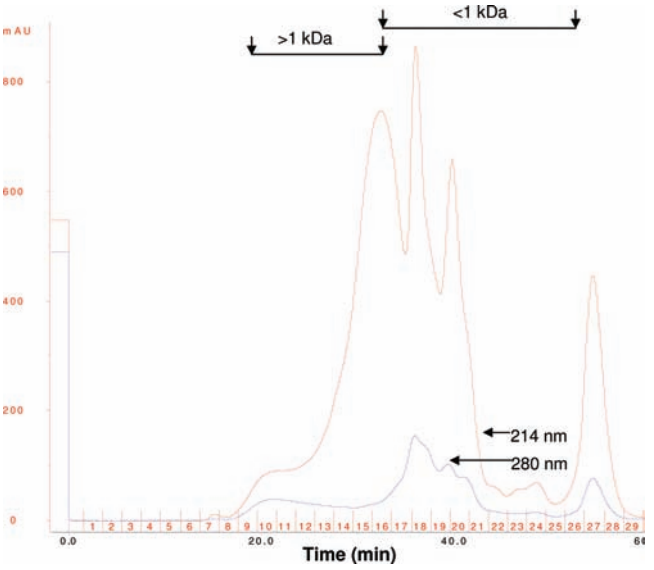

Figure 3. Molecular mass distribution profile of peptides in the gastric + small intestinal digest of flaxseed protein (SM).

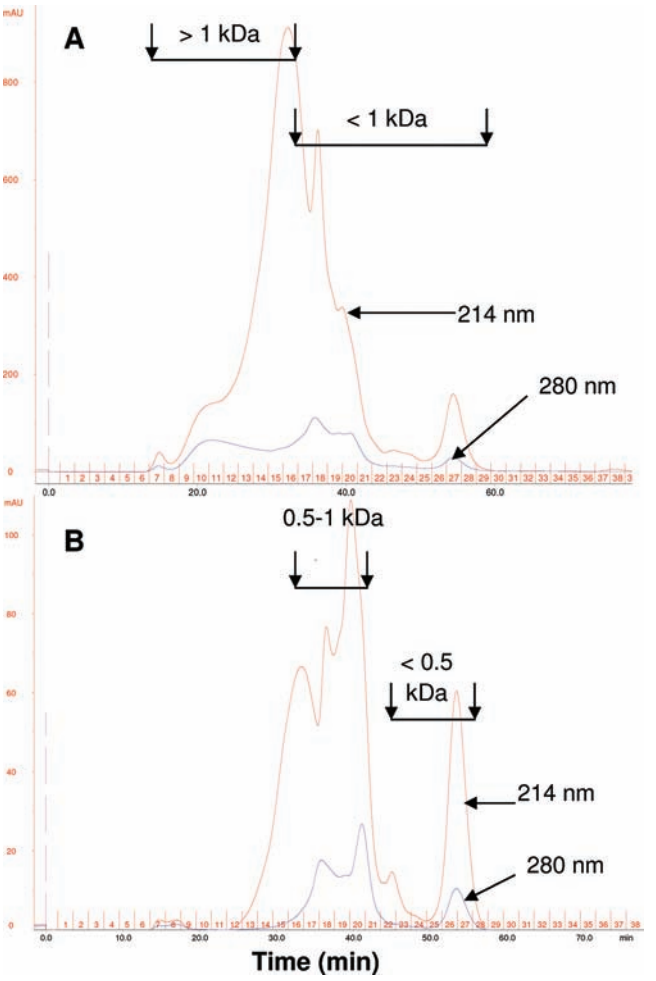

Figure 4. Molecular mass distribution profile of peptides in the (A) retentate and (B) dialysate of the gastric + small intestinal digest of flaxseed protein obtained using the DM.

than the gastric phase enzymes, generating a digest predominantly composed of dipeptides. Pepsin hydrolysis facilitates pancreatin digestion of proteins by increasing their solubility and opening the molecule's 3D structure, which improves the availability of peptide bonds to enzymes in the intestinal fluid.[31] Enzymes in pancreatin function collectively to cleave peptide bonds causing improved DH values. In contrast to the majority of the simulated GI studies for protein digestion,[11−13] in the current study, simulated bile was used in the intestinal phase along with SIF. Gas et al.[33] have reported the ability of conjugated bile acids to accelerate the activity of pancreatic proteases, which may have facilitated pancreatic digestion of flaxseed protein generating a digest with predominantly dipeptides.

The DH values of the gastric + small intestinal digest of the DM were calculated by taking the free amino groups in both dialysate and retentate into consideration. The higher DH shown by this digest of the DM as compared to that of the SM could be due to the absence of feedback inhibition of digestive enzymes by the accumulated products in the DM. However, even after 4 h of small intestinal digestion, DH indicated that mostly peptides were dominant as the flaxseed protein digestion products, rather than amino acids in free form.

Gastric digest as well as the gastric + small intestinal digest of flaxseed protein obtained using both SM and DM showed ACEI activity exhibiting the ability of flaxseed protein to release ACEI peptides during GI digestion. According to Cheung et al.,[9] the C-terminal residues of ACEI peptides play a predominant role in competitive binding to the active site of ACE. Peptides with hydrophobic and aromatic amino acids (Trp, Tyr, and Phe) at each of the three C-terminal positions as well as the imino acid, Pro, at the C-terminal are the most favorable for strong competitive binding to ACE.[34] Because pepsin cleaves at the carboxyl end of hydrophobic and aromatic amino acids (Phe, Tyr, Trp, and Leu), peptides with such amino acids at the C-terminal were expected to be the predominant products in the gastric digest. Such peptides bind tightly to ACE at its active site and compete with angiotensin I for occupancy; therefore, ACE cannot bind to angiotensin I and convert it to angiotensin II.[34]

The results of the SM showed that further hydrolysis by pancreatin in the intestinal phase had reduced the ACEI activity of peptides generated by pepsin assisted hydrolysis. Similar findings were reported by previous in vitro GI digestion studies,[10,12] which used SM for simulated digestion. In contrast to the SM, subsequent digestion with pancreatin improved the ACEI activity of the gastric + small intestinal digest in the DM as compared to the pepsin digestion alone. The significantly higher ($P < 0.05$) DH and presence of the aromatic amino acid, Tyr, in the gastric + small intestinal digests of the DM as compared to other digests indicated the possibility of having peptides with C-terminal Tyr in the gastric + small intestinal digest of the DM, which could have contributed to its ACEI activity.

Of the proteases in pancreatic secretions, chymotrypsin cleaves peptide bonds at the carboxyl end of aromatic and hydrophobic amino acids similar to pepsin, while pancreatic trypsin preferentially cleaves at the carboxyl end of arginyl and lysyl residues. Although the E/S used for pancreatin was similar in both models, the enzyme kinetics of pancreatin was expected to be different. The DM differed from the commonly used two-stage in vitro SM of digestion, in which the protein substrate was digested with pepsin first and then by pancreatin where absorption of digested products was not facilitated. Continuous

Table 5. ACEI Activity of Fractionated Dialysate of the Flaxseed Protein Digest (DM)[a]

fraction	molecular mass of peptide fraction (kDa)	ACEI activity (%)[b]	IC_{50} (mg of solids/mL)[b]
GIF-I	0.5−1	73.19 ± 0.75 b	3.71 ± 0.04
GIF-II	<0.5	1.36 ± 1.01 a	ND

[a] Means in the same column with different letters are significantly different ($p < 0.05$). ND, not determined. [b] Values are means ± standard deviations.

Table 6. Amino Acid Sequence of the Peptides in the Most Active ACEI-FPLC Fractions of the Dialysate of Flaxseed Protein Digest Obtained Using the DM

amino acid sequence[a]	calculated molecular mass[a]	probability[a]
Trp-Asn-Ile/Leu-Asn-Ala	616.30	91.31
Asn-Ile/Leu-Asp-Thr-Asp-Ile/Leu	689.32	100.00

[a] Obtained using the MassLynx software.

removal of digestion products during in vitro proteolysis represents a more realistic model of in vivo mechanism, where the epithelial brush border membranes remove the products of protein digestion from the lumen by independent mechanisms.[35] The SM of digestion leads to accumulation of hydrolysis products that may impede the progression of hydrolysis due to enzyme inhibition by products.[31] Dialysis used in the DM allowed simultaneous elimination of digested products and reduced the possibility of any enzyme inhibition by the generated products. It could have particularly enhanced the activity of pancreatic proteases especially chymotrypsin, which could be responsible for the improved ACEI activity of gastric + small intestinal digests. In the SM, the reduced protease activity of pancreatin caused by accumulated digestion products could have resulted in the hydrolysis of gastric phase generated peptides, degrading their amino acid sequence suitable to inhibit ACE. In addition, the results show that the commonly used two stage proteolysis model with no absorption for simulated GI digestion underestimates the ACEI activity of digested food protein.

A low IC_{50} is an indication of high ACEI potency. Simulated GI digestion studies (using digestion models with no absorption) have reported the IC_{50} for ACEI activity of gastric + small intestinal digests of soy protein (0.28 mg/mL; 12), chick pea (Desi), and yellow pea protein (0.14 and 0.16 mg/mL, respectively; 36), and pea protein isolate (0.07 mg/mL; 11), which was lower than that of the gastric + small intestinal digest obtained using the SM (IC_{50}, 0.39 mg N/mL or 0.69 mg solids/mL). However, the IC_{50} gastric + small intestinal digest (SM) of flaxseed protein was within the range reported for GI digested whey protein (0.35−1.73 mg/mL).[37] In the current study, the retentate and the dialysate of DM showed IC_{50} values of 0.05 and 0.04 mg N/mL, respectively. In a study conducted using a dynamic digestion model consisted of a dialysis membrane of 6−8 kDa MWCO, digested protein from common bean (IC_{50}, 0.83 mg protein/mL), pinto beans (IC_{50}, 0.69 mg protein/mL), and green lentils (IC_{50}, 0.89 mg protein/mL) showed lower ACEI activity[38] than that of the dialysate of flaxseed protein digest (DM) (IC_{50}, 0.04 mg N/mL, 0.23 mg of protein/mL, or 4.77 mg of solids/mL).

The dialysate of gastric + small intestinal digestion included absorbable peptides generated upon simulated GI digestion of flaxseed protein. Of the sequenced peptides in the most ACEI fraction (0.5−1 kDa) in the dialysate, Asn-Ile/Leu-Asp-Thr-Asp-Ile/Leu potentially contains C-terminal Leu, which was according to the cleavage specificity of chymotrypsin. The peptide Trp-Asn-Ile/Leu-Asn-Ala had C terminus Ala, which was not in agreement with the individual enzyme specificities in pancreatin. However, peptides with a diverse range of amino acids in the C-terminal position were expected in GIF-II due to simultaneous action of endopeptidases (trypsin, chymotrypsin, and elastase) and exopeptidases (carboxypeptidases A and B) in pancreatin. The C-terminal tripeptide sequence in the two peptides was compared to the structural requirement of ACEI peptides reported by Cheung et al.[9] Although not entirely hydrophobic or aromatic, both peptides had the hydrophobic amino acid Leu within the first three C-terminal positions. The structure−activity relationship of ACEI peptides is predicted mostly based on studies of di- and tripeptides, and they may not always be extrapolated to peptides with long PCL as detected in our study. The importance of C-terminal structure of peptides on ACEI potency is reduced when the PCL gets longer and other steric effects and conformation of peptides in a specific environment begin to interfere with the ACEI potency.[39]

To be physiologically active, ACEI peptides must reach the cardiovascular system in an active form.[40] Therefore, absorption of ACEI peptides through the intestinal epithelium plays an important role in determining their physiological functions. The dialysis membrane (1 kDa MWCO) of the DM of digestion selects free amino acids to peptides possibly 8−10 amino acids long, which is very close to the end products of intestinal digestion.[41] In the human gut, nutrients are transported by a transcellular and/or paracellular route from the intestinal lumen to the interstitial fluid and afterward to the blood. However, the DM only simulated the absorption of flaxseed protein digested products by passive diffusion that represents the paracellular pathway of peptide absorption. This is accomplished by passive diffusion, caused by the concentration gradient between the intestinal lumen and the interstitium.[42] The paracellular pathway keeps the transported peptide intact without further degradation. Of the peptides absorbed via the transcellular route, over 90% are further hydrolyzed in the absorptive cells by aminopeptidases, endopeptidases, and dipeptidases in the intestinal brush border into a mixture of amino acids and di- and tripeptides.[40] In the current study, intestinal brush border enzymes were not used; therefore, the fate of ACEI activity during transcellular passage of peptides cannot be predicted. Whichever the route, peptides even in small amounts could get into the body and demonstrate physiological functions.[43] None of the peptides identified in the dialysate were di- or tripeptides. Small peptides such as di- and tripeptides are easily absorbed through the intestinal epithelium. There are human studies reporting the intestinal absorption and antihypertensive activity of ACEI tripeptides, Val-Pro-Pro and Ile-Pro-Pro in sour milk.[44] In addition, there are studies reporting the bioavailability of long chain ACEI peptides that support our findings. For example, the ACEI peptides Ile-Pro-Pro-Leu and His-Leu-Pro-Leu-Pro were found in human plasma upon ingestion of a drink containing them.[45] ACEI peptides, His-Leu-Pro-Leu-Pro from β-casein,[46] Lys-Val-Leu-Pro-Val-Pro,[47] and

Gly-Ala-Hyp-Gly-Leu-Hyp-Gly-Pro (chicken collagen hydrolysate),[48] are transported through the intestinal epithelium intact via paracellular diffusion. Although a clear conclusion cannot be made, peptides in the retentate also could potentially be absorbed intact transcellularly, if not hydrolyzed by the brush border peptidases.

This is the first study reporting that the sequential digestion of flaxseed protein with GI tract enzymes is able to generate peptide fractions with high ACEI activity. None of the two peptides identified in the most ACEI dialysate fraction (0.5−1 kDa) matched with the amino acid sequence of flax conlinnin (2S; 19.06 kDa), which is the only flaxseed protein with a known amino acid sequence. Therefore, these peptides are probably derived from the flaxseed protein linin (12S). Further studies are needed to validate the ACEI activity of these peptides.

The present simulated GI digestion study showed that flaxseed protein generates peptides with ACEI activity, which may absorb via the intestinal epithelium, suggesting that the protein in flaxseed can contribute to its cardioprotective function. Controlled human studies are necessary to confirm these findings. Results of studies on bioactive peptide release during GI digestion obtained from the in vitro static digestion models should be interpreted with caution as values may be underestimated.

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ABBREVIATIONS USED

DH, degree of hydrolysis; PCL, peptide chain length; ACE, angiotensin 1-converting enzyme

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