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ORIGINAL PAPER

An In-vitro Investigation of Selected Biological Activities of Hydrolysed Flaxseed (*Linum usitatissimum* L.) Proteins

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Abstract A study was conducted to determine bioactivities of flaxseed (*Linum usitatissimum* L.; variety: Valour) proteins and their hydrolysates. Isolated flaxseed proteins were treated with Flavourzyme® at different levels of enzyme to substrate ratio (E/S) and hydrolysis time. The unhydrolysed proteins and hydrolysates were studied for angiotensin I-converting enzyme inhibiting (ACEI) activity, hydroxyl radical (OH·) scavenging activity and bile acid binding ability. Flavourzyme catalysed hydrolysis generated hydrolysates with a 11.94–70.62% degree of hydrolysis (DH). The hydrolysates (0.67 mg/ml) had strong ACEI activity (71.59–88.29%). The maximum ACEI activity containing hydrolysate exhibited an IC_{50} of 0.07 mg/ml (E/S: 1.5; Time: 12 h; DH: 11.94%). The OH· scavenging activity of the hydrolysates (0.5 mg/ml) was 12.48–22.08% with an IC_{50} of 1.56 mg/ml in the sample possessing maximum activity (E/S: 47.5; Time 0.7 h; DH: 24.63%). Both these activities were greater in hydrolysates with lower DH and higher peptide chain length (PCL) than those with higher DH and lower PCL. Hydrolysed flaxseed proteins (0.67 mg/ml) had no bile acid binding ability. The unhydrolysed proteins had no ACEI or OH· scavenging activity but demonstrated bile acid binding ability.

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Introduction

Flaxseed (*Linum usitatissimum* L.) is an important oilseed in cool climatic regions. Canada is the world leader in the production and export of flaxseed. In year 2005/2006, total Canadian flaxseed production was 1.035 MT and about 50% of this was exported [1]. Whole flaxseed is rich in lipids, dietary fibre (both soluble and insoluble), proteins and lignans [2]. The major product obtained from flaxseed is oil, which is the richest known plant source of the omega 3 (ω-3) fatty acid, α-linolenic acid (ALA). The residual meal that remains after oil extraction has 35–40% protein and has traditionally been used in animal feed [3]. Currently, the defatted meal is marketed for food applications as a convenient source of dietary fibre, lignans, magnesium, zinc and soluble fibre [4]. However, the proteins of flaxseed meal have not yet been widely exploited for their value in human nutrition.

Major storage proteins of flaxseed are linin (11–12 S) and conlinin (2 S) that have molecular masses of 252–298 and 16–17 kDa, respectively [2, 5]. The amino acid composition of flaxseed proteins is comparable to that of soy with both seeds having relatively high levels of aspartic acid, glutamic acid, leucine and arginine [2]. At present, flaxseed proteins have not been well characterised and most of the properties of its protein require further investigation.

Currently, flaxseed is receiving much attention as a functional food among consumers and health care professionals due to the reported health benefits of its components. Both whole flaxseed and its meal have

demonstrated protection against cardiovascular disease (CVD) and several other diseases [6]. The specific component of flaxseed, which contributes to these health benefits, has not been completely elucidated. Most of the research to investigate the role of flaxseed on human health has focused on ALA-rich flaxseed oil, flaxseed lignans and soluble polysaccharides whereas its protein constituent has received limited attention. Only limited information is available on potential health benefits of flaxseed proteins.

The role of dietary proteins as physiologically active components beyond the classical nutritive value has been increasingly acknowledged in recent years. It is well accepted that 30–50% of dietary nitrogen is absorbed into the body as small peptides, which can also exert different bioactivities [7]. Therefore, currently significant attention is given to study and understand the ability of food proteins to generate bioactive peptides. Bioactive peptides are the specific protein fragments that have positive impact on body functions or conditions and which ultimately influence body health [8]. These peptides are inactive within the sequence of parent proteins and can be released during gastrointestinal digestion or food processing upon which they exert various physiological functions based on the inherent amino acid composition and sequence. At present, several enzymatically hydrolysed seed storage proteins have been studied for the presence of bioactive peptides possessing angiotensin I-converting enzyme inhibitory (ACEI) activity (antihypertensive), antioxidant, opioid, bile acid binding, immunostimulating, mineral utilising and antimicrobial activities [8, 9]. Such protein hydrolysates will provide potential ingredients in formulating functional foods and nutraceuticals that can be protective against human diseases. Among chronic human diseases, CVD is the leading cause of mortality and morbidity in the world. Hypertension, oxidative stress caused by free radicals and high blood cholesterol level are some of the factors that increase the risk of CVD. The therapeutic interventions with CVD include angiotensin I-converting enzyme (ACE) inhibitors, which regulate blood pressure, antioxidative agents and cholesterol lowering drugs [10]. Identifying nutraceuticals or functional food ingredients that can be used for intervention to major diseases will be very important.

A major problem in the hydrolysis of proteins by proteases is the development of bitter tasting peptides. Flavourzyme is a complex of exopeptidases and endoproteases, which causes extensive hydrolysis of food proteins cleaving off the hydrophobic residues and avoiding the formation of peptides with bitter flavours [11]. This fungi-origin enzyme complex has been used to provide peptides with short chain length; i.e. high degree of hydrolysis (DH) from storage proteins such as soybeans [12]. Use of Flavourzyme as the hydrolysing enzyme is advantageous over the use of other commercially available fungal proteases, which are mainly endoproteases that tend to generate bitter tasting peptides.

To the best of our knowledge, published information on bioactive peptides derived from hydrolysed flaxseed protein is limited except for the work of Omoni and Aluko [13]. They reported in-vitro calmodulin binding activity and calmodulin dependent neuronal nitric oxide synthase inhibitory activity of Alcalase-hydrolysate of flaxseed. Wu et al. [14] reported in a patent that proteolytic digestion of defatted flaxseed meal (without purifying its protein) generates a hydrolysate with ACEI activity. The objective of the present research was to prepare flaxseed proteins in isolated form and to investigate the ACEI activity, hydroxyl radical (OH·) scavenging activity and bile acid binding ability of the Flavourzyme® hydrolysed proteins. Degradation of flaxseed proteins due to the hydrolytic conditions provided was also investigated.

Experimental Procedures

Materials

The flaxseed (variety; Valour) used in this study was from a seed supplier in Saskatoon, Canada. The ready gels used for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were from General Electric Health Care, Amersham Pharmacia. Flavourzyme [from *Aspergillus oryzae*; EC NO. 232-752-2; 554.0 leucine amino peptidase units (LAPU)/g], *o*-Phthalaldehyde (OPA), L-serine, DL-dithiothreitol, sodium tetra borate decahydrate, ACE from porcine kidney (35 U/mg protein), hippuryl-histidyl-leucine (HHL), captopril, 2-deoxy-D-ribose (DR), hippuric acid, L-ascorbic acid, 2-thiobarbituric acid (TBA), hydrogen peroxide, L-carnosine, sodium cholate (SC), sodium taurocholate (STC), sodium glycocholate (SGC), sodium deoxycholate (SDC), sodium chenodeoxycholate (SCDC) and cholestyramine resin were purchased from Sigma Chemical Co. (St Louis, MO, USA). The total bile acid assay kit was purchased from Bio-Quant (San Diego, CA, USA). The total dietary fibre assay kit was purchased from Megazyme International Ireland Ltd (Wicklow, Ireland). Ferric chloride anhydrous, 98% was from Alfa Aesar (Ward Hill, MA, USA). Trichloroacetic acid (TCA) was from Merck (Germany). HPLC grade acetonitrile was from EMD chemicals (Gibbstown, NJ, USA). All the reagents used for the experiments were of analytical grade. All chemical assays were carried out as triplicate determinations.

Proximate Analysis

The proximate composition (moisture, ash, protein and fat) of whole flaxseeds and the isolated protein was analysed

according to AOAC [15] procedures. The soluble carbohydrate levels were estimated by analysing sugars in a water extract using the phenol-sulfuric method [16] with L-rhamnose as the standard. The total dietary fibre content was analysed using Megazyme total dietary fibre assay kit.

Extraction and Isolation of Proteins from Flaxseed

Flaxseed protein extraction was carried out according to Wanasundara and Shahidi [17] with modifications. The major modifications included the use of an oil expeller for de-oiling and the use of NaOH for protein extraction. Mucilage in the seed coat of flaxseed was removed (demucilaging) and de-oiling was carried out on demucilaged seeds prior to protein extraction. To remove mucilage, flaxseeds were stirred with 0.5 M $NaHCO_3$ (1:8 w/v, 50 °C) for 1 h and then seeds were recovered by filtration. The seeds were then manually rubbed against an aluminium wire mesh and washed thoroughly with distilled water. The extraction and washing steps were completed two more times. After draining and drying in an oven at 45 °C for 24 h, the demucilaged flaxseeds were de-oiled using an oil expeller (screw press). The resulting meal was ground using a home-style coffee grinder, passed through a 500 μm screen and subjected to further de-oiling by stirring with *n*-hexane (1:3, w/v) at room temperature for 6 h with renewal of hexane every 2 h. The resulting flaxseed meal was air dried, ground, sieved and used for protein extraction.

The meal proteins were extracted by alkali solubilisation. Flaxseed meal was suspended in distilled water and the pH was adjusted to 8.5 using 1 M and 0.1 M NaOH solution. The final volume of the mixture was adjusted to have a meal to alkali solution ratio of 1:10 (w/v). The mixture was stirred for 1 h on a magnetic stirring plate at room temperature to extract protein. The alkali extract was centrifuged at 8,820×*g* for 20 min to recover both the supernatant and the residue. The residue was subjected to two more extractions (residue:water 1:10 w/v at pH 8.5, for 1 h) followed by centrifugation as above. The proteins of the pooled supernatants were precipitated at pH 3.8 (using 0.1 M HCl) according to the findings of preliminary experiments, and were separated by centrifugation (8,820×*g* for 20 min). Recovered precipitate was washed once with distilled water to remove excess acid. The protein precipitate was slurried in distilled water and pH was adjusted to 7.0 using 0.1 M NaOH. The protein solution was then dialysed against distilled water using a dialysis membrane (Spectra/Por, MWCO 3500) at 4 °C for 48 h to remove any small molecules such as salts and then lyophilised. The supernatant remained after protein precipitation was also recovered and lyophilised. The freeze-dried flaxseed protein isolate was stored at 4 °C until needed.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis of Proteins

Separation of proteins of flaxseed meal, precipitated or isolated protein, soluble fraction (protein that was not precipitated by acid) and the indigestible residues of isolated protein remaining upon Flavourzyme catalysed hydrolysis (unhydrolysed residue) was carried out according to Laemmli procedure [18]. β-Mercaptoethanol (β-ME) was not included in the sample buffer when non-reducing conditions were required. Gradient mini gels (resolving 8–25%T and 2%C, stacking zone 4.5%T and 3%C, 43 mm × 50 mm × 0.45 mm, polyacrylamide gels cast on GelBond® plastic backing, buffer 0.112 M acetate, 0.112 M Tris, pH 6.4) were used to separate proteins on a PhastSystem equipped with separation and development capabilities (Amersham Pharmacia, Uppsala, Sweden). Sample of 1 μg protein was applied into each well and standard proteins were also applied into a separate well. The molecular weight standards (Sigma wide range molecular weight markers) ranging from 6.5 to 205 kDa and 11 to 170 kDa were used to estimate size of the proteins in samples. Following separation, the proteins were fixed and stained using PhastGel blue R (Coomassie R-350) and developed to obtain suitable background colour.

Enzymatic Hydrolysis of Flaxseed Proteins

The hydrolysis of isolated flaxseed proteins was carried out according to a two factor central composite rotatable design (CCRD) using nine combinations of enzyme: substrate (E/S; LAPU/g of protein) and hydrolysis time (h) as presented in Table 1. A 2% (w/v) slurry of the isolated

Table 1 Hydrolysis conditions employed for isolated flaxseed proteins and the resultant degree of hydrolysis (DH%) and peptide chain length (PCL)

E/S, LAPU/g protein[a]	Time of hydrolysis[a] h	DH %	PCL
1.5	12.0	11.94 ± 4.1	9.16 ± 3.2
15.0	4.0	25.13 ± 1.6	3.99 ± 0.3
15.0	20.0	47.30 ± 3.5	2.12 ± 0.2
47.5	0.7	24.63 ± 2.9	4.11 ± 0.5
47.5	12.0	64.24 ± 0.7	1.56 ± 0.02
47.5	23.3	65.42 ± 5.1	1.54 ± 0.1
80.0	4.0	57.15 ± 4.1	1.76 ± 0.1
80.0	20.0	70.62 ± 5.9	1.42 ± 0.1
93.5	12.0	64.86 ± 4.7	1.55 ± 0.1

E/S enzyme to substrate ratio, *LAPU* leucine amino peptidase units

[a] Experimental points obtained using a central composite rotatable design. Temperature was maintained at 50 °C

proteins in deionised water was adjusted to pH 7.0 using 0.1 M NaOH solution and hydrolysed using the fungal enzyme Flavourzyme (declared activity of 554 LAPU/g). The reaction mixture was continuously stirred in a double-jacketed spinner flask, on a magnetic stir plate and connected to a circulating water bath at conditions required for the optimum activity of Flavourzyme (50 °C, pH 5–7) as defined by the supplier. Hydrolysis was terminated by heat (95 °C, 10 min) inactivation of the enzyme. The hydrolysed protein solution was centrifuged at 8,820×*g* for 15 min to recover non-solubles (unhydrolysed residue) and the supernatant (hydrolysate). The hydrolysates were freeze-dried and stored at −20 °C. The unhydrolysed residue recovered as pellets was stored at −70 °C until used. Each hydrolysis experiment was carried out a minimum of two times.

Estimation of Total Nitrogen and Protein Content

The nitrogen content of flaxseed meal, isolated protein and hydrolysates were analysed by combustion N analysis using a Thermo Erba N/protein analyzer. Nitrogen values were converted to protein using 6.25 as the conversion factor when needed. The protein content of unhydrolysed residues that remained after hydrolysis of flaxseed protein was determined according to the Bradford method [19].

Estimation of Degree of Hydrolysis and Average Peptide Chain Length

The DH of the protein was determined using available free amino groups upon hydrolysis, according to the OPA method as described by Wanasundara et al. [20]. The OPA reagent, prepared daily, consisted of 6 mM OPA (first dissolved in 95% v/v ethanol) and 5.7 mM DL-dithiothreitol in 0.1 M sodium tetraborate decahydrate containing 2% (w/v) SDS. Samples (0.4 ml) were mixed with 3 ml of OPA reagent, vortexed and incubated for 2 min at room temperature before measuring the absorbance at 340 nm using a HP-8453 diode array spectrophotometer. The amount of free amino groups in the hydrolysate was calculated as serine-NH_2 moieties using L-serine as the standard. Total number of primary amino groups in the flaxseed protein was determined by acid hydrolysis of flaxseed protein (6 N HCl at 110 °C for 24 h) assuming a complete hydrolysis of all peptide bonds of the protein was achieved during acid digestion. The % DH was calculated according to the Eq. 1.

$$\%\mathrm{DH} = \frac{[\mathrm{NH_2}]_{Tx} - [\mathrm{NH_2}]_{To}}{[\mathrm{NH_2}]_{\mathrm{Total}} - [\mathrm{NH_2}]_{To}} \times 100 \quad (1)$$

$[\mathrm{NH_2}]_{To}$ = Number of free -NH_2 groups at 0 min of hydrolysis

$[\mathrm{NH_2}]_{Tx}$ = Number of free -NH_2 groups in the supernatant after *x* minute of Flavourzyme catalysed hydrolysis for each experimental point

$[\mathrm{NH_2}]_{\mathrm{Total}}$ = Number of -NH_2 groups resulting due to acid hydrolysis (complete hydrolysis of protein is assumed).

The average peptide chain length (PCL) in the hydrolysates was calculated according to the Eq. 2 given below [21]

$$\text{Average peptide chain length} = \frac{100}{\mathrm{DH}} \quad (2)$$

Determination of In-vitro Bioactivities of the Flaxseed Protein Hydrolysates

ACE Inhibitory Activity

Angiotensin I-converting enzyme inhibitory activity of the hydrolysed flaxseed proteins was determined according to the method reported by Wanasundara et al. [22] with some modifications. HHL was used as the substrate for ACE. The assay was carried out in Tris-buffer (100 mM Tris–HCl, 300 mM NaCl, 10 μM $ZnCl_2$ in deionised water; pH 8.3), which was used to dilute the enzyme, substrate and the inhibitors. The initial assay mixture consisted of 50 μl of 3 mM HHL, 50 μl of ACE (from porcine kidney, containing 1.25 milliunits of declared enzyme activity) and 50 μl of the hydrolysate (0.67 mg/ml in the final assay mix). The reaction components were incubated in glass vials at 37 °C for 30 min in a water bath before and after mixing. Addition of glacial acetic acid (150 μl) to the reaction mixture terminated the reaction. The free hippuric acid in the reaction mixture (filtered using 0.2 μm filters) liberated due to action of ACE on HHL was determined using HPLC with a reversed-phase C18 column (Bond clone C18, 5 μ, 250 mm × 4.6 mm) protected by a guard column (Bond clone C18, 10 μ, 50 mm × 1.0 mm; Phenomenex, Torrance, CA, USA). The HPLC system consisted of a solvent delivery system pump (Agilent 1100 series), an auto sampler, a diode array LC detector and was supported by Chemstation A 10.01 software. The isocratic mobile phase used for elution of hippuric acid consisted of 25% (v/v) acetonitrile in deionised water, with pH 3.0 adjusted with glacial acetic acid. The injection volume used was 10 μl with a flow rate of 1 ml/min. Eluted hippuric acid was detected by monitoring the absorbance at 238 nm. A standard curve was constructed using a series of hippuric acid standards of known concentration to quantify the released hippuric acid in the assay mix. Captopril (0.67 mg/ml in Tris-buffer), which is the clinical inhibitor of ACE, was used for comparative purpose. The control reaction mixture was composed of all the reaction components except the hydrolysate, which was replaced by

50 µl of Tris-buffer and was expected to release maximum amount of hippuric acid from HHL due to uninhibited ACE activity. The percent inhibition of ACE activity was calculated using Eq. 3.

$$\%\text{ACE inhibition} = \frac{(\text{Hippuric acid})_{(\text{Control})} - (\text{Hippuric acid})_{(\text{Sample})}}{(\text{Hippuric acid})_{(\text{Control})}} \times 100 \quad (3)$$

Hydroxyl Radical Scavenging Activity

The ability of the hydrolysates to scavenge OH· was determined using DR assay, originally described by Halliwell et al. [23] with some adaptations. In this assay, DR is oxidised by OH· that is generated from the Fenton reaction, into malonaldehyde, which can be quantified by its condensation with TBA [23]. The ability of hydrolysates to suppress breakdown of DR into 2-TBA reactive substances via scavenging OH· was determined. The freeze-dried hydrolysates were dissolved in potassium phosphate buffer (0.5 mg/ml of final assay, pH: 7.4) prior to the assay. The following components were sequentially added to a glass test tube to form 1 ml of the final reaction mix; 400 µl of 0.05 mM $FeCl_3$, 42 µl of 2.4 mM EDTA, 142 µl of 1 mM H_2O_2, 151 µl of potassium phosphate buffer, 14 µl of 0.2 M DR in water, 250 µl of hydrolysate (0.5 mg/ml) and 1 µl of 0.1 M ascorbic acid solution. The assay mix was incubated at 37 °C in a water bath for 1 h. One millilitre of 1% (w/v) 2-TBA and 1.0 ml of 2.8% (w/v) TCA were added to the test tubes after 1 h and incubated at 100 °C for 20 min. After cooling to room temperature the absorbance of the assay mix was measured at 532 nm against a blank, which consisted of all the reagents, to which TBA and TCA were added prior to incubation at 37 °C. A control reaction containing potassium phosphate buffer as a replacement for the hydrolysate was also performed. Carnosine (0.5 mg/ml), a naturally occurring antioxidant dipeptide, was used as the standard. The OH· scavenging activity was determined using the following Eq. 4.

$$\%\text{OH·scavenging activity} = \frac{\left[(A_{532})_{\text{Control}} - (A_{532})_{\text{Sample}}\right]}{(A_{532})_{\text{Control}}} \times 100 \quad (4)$$

A_{532} is the absorbance at 532 nm.

Bile Acid Binding Ability

The in-vitro bile acid binding capacity of isolated flaxseed protein and hydrolysates was assessed as described by Yoshie-Stark and Wasche [9]. The bile acids tested were SC (primary), SGC (secondary), STC (secondary), SDC (secondary) and SCDC (primary). The test samples were dissolved in 0.1 M sodium phosphate buffer (pH 7.0) at a concentration of 6.7 mg/ml, prior to the assay. One hundred micro litres of sample suspension was mixed with 900 µl of 2 mM bile acid solution in sodium phosphate buffer in a micro-centrifuge tube. The mix was incubated at 37 °C for 2 h, centrifuged (16,000×g for 5 min); the supernatant was recovered and transferred into a 25-ml volumetric flask. The remaining residue was mixed with 500 µl of sodium phosphate buffer and centrifuged again. The supernatants were pooled in the volumetric flask and the final volume was adjusted to the mark using sodium phosphate buffer. A control reaction mixture consisting of 100 µl of sodium phosphate buffer and 900 µl of 2 mM bile acid solution was incubated along with samples and treated similarly. Bio-Quant total bile acid analysis kit was used to quantify free bile acids in the supernatants. The blank consisted of all the reagents used for final spectrophotometric analysis except the test sample, which was replaced by the sodium phosphate buffer. All the bile acids were monitored at 530 nm. Calibration curves were constructed for each of the bile acid tested. Cholestyramine resin (0.67 mg/ml), which is the clinically approved bile acid binding agent, was used as the standard. The bile acid binding ability was determined as the % bile acid bound using Eq. 5. Binding of each bile acid was tested separately.

$$\%\text{Bile acid binding} = \frac{\left[(A_{530})_{\text{Control}} - (A_{530})\right]_{\text{Sample}}}{(A_{530})_{\text{Control}}} \times 100 \quad (5)$$

A_{530} is the absorbance at 530 nm.

Determination of IC_{50} Values of the Bioactivities

The IC_{50} value was defined as the concentration of hydrolysate (mg/ml) required to achieve 50% of the control value for the bioactivities. The IC_{50} values were calculated for each of the bioactivities tested by selecting the hydrolysates possessing maximum of the tested bioactivities.

Statistical Analysis

Data obtained from the hydrolysis experiment (arranged as a CCRD) was analysed according to response surface methodology. In this analysis ANOVA and *t*-test was employed to test the significance (at 0.05 probability level) of the hydrolysis time and E/S on %DH and PCL. Values are reported as average ± standard deviation. Statistical analysis was performed using SAS 9.1 software.

Results and Discussion

Composition of Flaxseeds and Isolated Proteins

Flaxseed (variety: Valour) contained 5.74 ± 0.1% moisture, 4.04 ± 0.0% ash, 40.62 ± 0.5% fat, 22.18 ± 0.3% protein (N% × 6.25) and 12.16 ± 0.6% soluble carbohydrates on a fresh weight basis. Demucilaging and de-oiling of flaxseeds was carried out prior to protein extraction as both mucilage and oil seriously interfere with the extraction and recovery of protein [24]. The estimate of soluble carbohydrate content gives an indirect measurement for the mucilage content [25] and mucilage reduction treatment removed 82.40% of total soluble carbohydrates of seed coat. According to our preliminary studies on pH vs. protein solubility, the minimum soluble protein level from flaxseed meal was identified at pH 3.8. Therefore during protein extraction the pH of alkali solubilised flaxseed meal was adjusted to 3.8 to precipitate and recover the maximum amount of proteins. The flaxseed protein isolated using this method had 80.19 ± 0.6% (N% × 6.25) protein along with 3.35 ± 0.1% ash, 0.26 ± 0.004% fat and 8.63 ± 0.3% dietary fibre on a dry weight basis.

Hydrolysis of Flaxseed Proteins

The isolated flaxseed protein was hydrolysed by Flavourzyme at different E/S and time of hydrolysis. Flavourzyme caused extensive hydrolysis (>50% DH) of flaxseed protein under the majority of the E/S and hydrolysis time combinations used (Table 1). The N content of the lyophilised hydrolysates ranged from 11.18 to 12.51%. The DH, which is a measure of the number of peptide bonds cleaved as a percentage of total peptide bonds in the protein [21], ranged from 11.94 to 70.62% (Table 1) for flaxseed protein and increased with the increasing E/S and time of hydrolysis. The time of hydrolysis and E/S had significant ($p < 0.01$) effect on DH values. The maximum DH (70.62%) was achieved when the protein was hydrolysed at an E/S of 80 (LAPU/g of protein) for 20 h. The PCL of the hydrolysates ranged from 1.4 to 9.2 and decreased with the increasing DH (Table 1). Average PCL of the majority of the hydrolysates was <4 indicating extensive hydrolysis of flaxseed protein had occurred. Only the E/S had a significant ($p < 0.01$) effect on PCL. There is no previously published research on Flavourzyme catalysed hydrolysis of flaxseed proteins. The reported DH of Flavourzyme hydrolysed proteins from other oilseeds such as soy (DH: 39.5% upon 8 h of hydrolysis) [12] and sunflower (DH: 42.2% upon 3 h of hydrolysis) [26] are lower than the maximum DH exhibited by flaxseed protein in this study, which may be due to the differences in the protein composition, time of hydrolysis, enzyme to substrate ratios employed and differences in sensitivity of the methods used for determining DH.

Figure 1a shows the SDS-PAGE analysis of flaxseed meal, isolated proteins and supernatant protein with and without disulfide (S–S) bond reduction. The polypeptide profiles of precipitated protein at pH 3.8 were not much different than the intact proteins of the meal or soluble (supernatant) fraction at pH 3.8 indicating isolated proteins represent the meal protein composition. All the polypeptide bands of the isolate recovered as acid precipitate were below 55 kDa in molecular mass. Under non-reducing conditions flaxseed protein appeared as four major bands; two distinct bands between 36 and 55 kDa and the other two between 6.5 and 14 kDa. There were several other minor bands present besides these. However, under reducing conditions the protein bands of high molecular mass (36–55 kDa) disappeared and were replaced by three major bands, each between 29 and 36 kDa, 20 and 24 kDa and below 14 kDa. This change was due to dissociation of

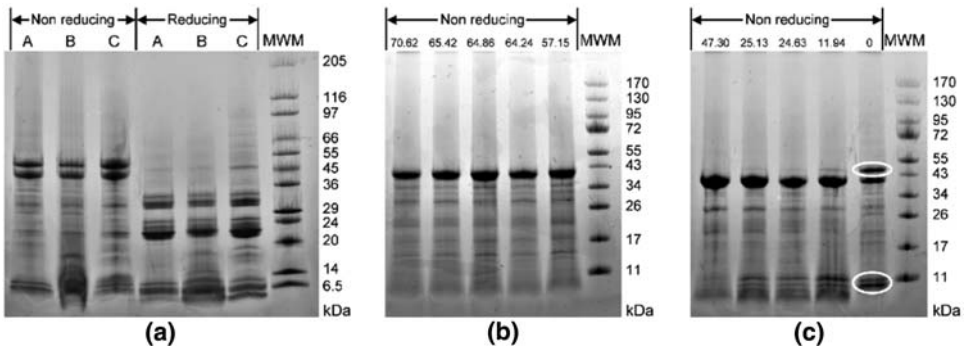

Fig. 1 **a** Electrophoretic separation (Tris-Tricine buffer) of isolated flaxseed proteins and meal under non-reducing and reducing conditions. *Lane A*: protein precipitate at pH 3.8; *lane B*: Soluble (supernatant) proteins at pH 3.8; *lane C*: Flaxseed meal; *MWM* molecular weight markers. **b, c** are electrophoretic separation (Tris-Tricine buffer) of flaxseed protein isolate and residues remaining after hydrolysis under non-reducing conditions. Lanes are labelled with DH%; 0 DH indicates unhydrolysed flaxseed protein isolated at pH 3.8; *MWM* molecular weight markers

S–S linked polypeptides caused by β-ME activity. Chung et al. [5] determined the disulfide content of flaxseed proteins as 121 μmol/g of protein, which is greater than that of soy proteins. This suggests that in flaxseed proteins, the polypeptide bands with high molecular masses are oligomers (36–55 kDa) with disulfide-bonded (S–S) polypeptides, which dissociates into free polypeptides in the presence of reducing agent. There was no major difference in the polypeptide bands of low molecular mass range (6.5–14 kDa) due to S–S bond reduction. Sammour [3] identified the occurrence of six major polypeptide bands (MW: 41, 43, 45 and 47, 50 and 55 kDa) in flaxseed (cultivar: Viking) proteins under non-reducing conditions. The difference in results could be due to different varieties of flaxseed used as the composition of proteins could differ depending on the cultivar or variety of flaxseed.

The SDS-PAGE patterns (non-reducing) of the protein isolate residues that remained upon Flavourzyme catalysed hydrolysis are shown in Fig. 1b, c. Out of the four major protein bands observed in the isolated flaxseed protein, the band with a molecular mass of around 34–43 kDa was visible in all the residues indicating its resistance to Flavourzyme hydrolysis. The high molecular weight protein band between 43 and 55 kDa and two low molecular weight protein bands around 11 kDa showed clear staining intensity decrease due to hydrolysis (Fig. 1c encircled protein bands). The high molecular weight protein band disappeared after DH value of 11.94%, while the low molecular weight bands disappeared after 25.13% DH. There were new polypeptide bands that appeared with apparent mass between 43 and 11 kDa, most likely the hydrolytic products of the parent protein of the isolate. Results indicate that flaxseed proteins <43 kDa mass were less susceptible to Flavourzyme catalysed hydrolysis when compared to the protein bands between 43 and 55 kDa. The indigestible residues corresponding to DH between 57.15 and 70.62% showed identical electrophoresis patterns with 34–43 kDa protein bands as the major remaining protein.

ACE Inhibitory Activity

The freeze-dried, isolated flax proteins and unfractionated (crude) protein hydrolysates were analysed for in-vitro bioactivities, namely ACEI activity, OH· scavenging activity and bile acid binding ability. The ACEI activity represents antihypertensive activity. ACE raises blood pressure by catalysing the conversion of the decapeptide angiotensin I into potent vasoconstricting octapeptide angiotensin II together with inactivating the vasodilator bradykinin, a blood pressure lowering nanopeptide [27]. Various synthetic ACE inhibitors are currently in use as antihypertensive agents whereas ACEI peptides have been reported from enzymatic digests of food proteins [28]. The in-vitro ACEI assay used in the present study was based on the ability of ACE to hydrolyse and release hippuric acid from the substrate, HHL [27]. ACE inhibitors if present in the assay mix, bind to the active site of ACE and inhibit the conversion of HHL into hippuric acid. Hippuric acid liberated in the ACE assay mix was eluted at a retention time of 5 min under the HPLC conditions used. Presence of flaxseed protein hydrolysates reduced the liberation of hippuric acid from HHL due to reduction of the activity of ACE indicating the presence of ACE inhibitors in the hydrolysates. The % ACEI activity of the crude flaxseed protein hydrolysates (0.67 mg hydrolysate/ml in Tris-buffer) ranged from 71.59 to 88.29% indicating that hydrolysates have more than 70% ACEI activity. Maximum ACEI activity (88.29%) was observed when the protein was hydrolysed for 12 h at E/S of 1.5 LAPU/g protein. The isolated proteins at unhydrolysed state had no ACEI activity suggesting that the observed ACEI activity was due to peptides generated during protein hydrolysis. A reduction in the intensity of protein bands around 43–55 kDa and 11 kDa occurred when the DH achieved 11.94% (Fig. 1). Therefore, it could be suggested that these two protein bands may act as precursor proteins for generation of ACEI peptides in hydrolysed flaxseed protein. A significant ($p < 0.05$) decrease in this activity was observed in hydrolysates when DH was beyond 25% (Fig. 2), indicating extensive hydrolysis that resulted in low PCL (below 3.99) and reduced ACEI activity. Wu et al. [29] reported that in long chain peptides (4–10 amino acid residues), the tetra peptide residue at the C-terminal end determines their structural requirement for effective

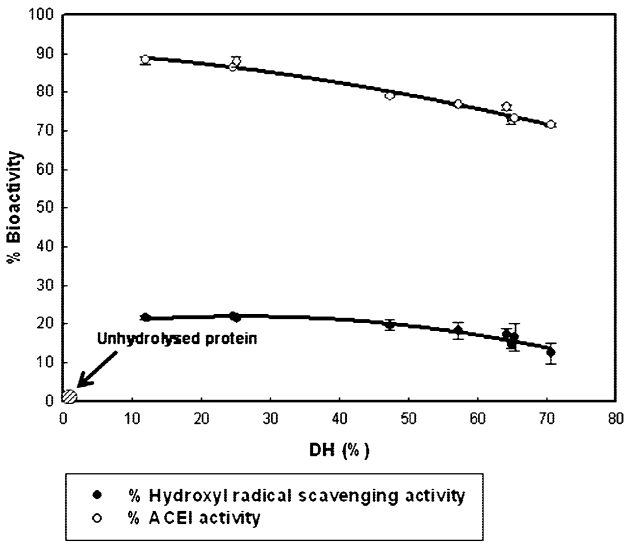

Fig. 2 Angiotensin I-converting enzyme inhibiting activity and hydroxyl radical OH· scavenging activity of hydrolysed flaxseed protein in relation to DH ($r^2 = 0.98$ and 0.94 respectively. Concentration of 0.5 and 0.67 mg hydrolysate/ml was used for the assays)

inhibition of ACE activity. Therefore, it could be hypothesised that with the increase of DH%, the exoproteases in Flavourzyme may be involved in removing amino acids from the C-terminal or N-terminal position of the ACE inhibitory peptides making them less active resulting in a decrease in ACEI activity, as DH is increased further. However, the lowest percentage of ACE inhibition observed in this study was 71.59% (at 70.62% DH), which is relatively high ACEI activity for a crude hydrolysate. The clinical ACEI drug Captopril (0.67 mg/ml) had 100% ACE inhibition under the in-vitro conditions used. Three hydrolysates possessing ACEI activity of over 85% gave the IC_{50} values ranging between 0.07 and 0.09 mg/ml (Table 2) suggesting the ability of hydrolysates to inhibit 50% of ACE activity at a low concentration. The ACEI IC_{50} values observed in the present study are greater than the values reported for defatted flaxseed meal hydrolysed with Alcalase and Thermolysin [14]. According to the study of Wu et al. [14], Alcalase hydrolysis of defatted flaxseed meal produced a hydrolysate with an IC_{50} of 64.3 μg/ml for ACEI, whereas the hydrolysate produced by Thermolysin had an IC_{50} of 37.1 μg/ml. The hydrolysate resulting from sequential hydrolysis of Thermolysin and Alcalase had an IC_{50} of 34.2 μg/ml for ACEI. However, ACEI IC_{50} of the flaxseed protein hydrolysate was much lower compared to the reported values for crude hydrolysates of soy protein hydrolysed with Alcalase (IC_{50} 0.34 mg/ml) [30] and rapeseed protein hydrolysed with thermolysin (IC_{50} 0.32 mg/ml) [31].

Hydrolysis of protein meal results in hydrolysates that contain various soluble, non protein matrix components. For example, strong ACEI activities have been reported from plant derived flavonoids and proanthocyanidins [32, 33] and guanosine derivatives [34]. In the present study, isolated flax protein in the unhydrolysed state did not show any ACEI activity. However, when treated with a proteolytic enzyme, the resulting hydrolysates exhibited ACEI activity. This observation indicates proteolytic degradation generated compounds (peptides) that have ACEI activity.

Table 2 IC_{50} of hydrolysates for ACEI and OH· scavenging activities

Type of bioactivity	DH %	Activity %	IC_{50} (mg solid/ml)
ACEI activity	11.94	88.29	0.07 ± 0.0 (0.0004)
	24.63	86.45	0.09 ± 0.0 (0.003)
	25.13	87.83	0.07 ± 0.0 (0.01)
OH· scavenging activity	11.94	21.63	3.06 ± 2.4
	24.63	22.08	1.56 ± 0.8
	25.13	21.46	2.03 ± 0.5

DH % degree of hydrolysis as a %, *ACEI* angiotensin I-converting enzyme inhibitory activity, OH· hydroxyl radical

OH· Scavenging Activity

The crude flaxseed protein hydrolysates were also tested for in-vitro OH· scavenging activity, which represents antioxidant activity. Unhydrolysed proteins did not show OH· scavenging activity. The OH· scavenging activity of the flaxseed protein hydrolysates (0.5 mg/ml) ranged from 12.48 to 22.08% (Fig. 2). The maximum OH· scavenging activity (22.08%) was found in the hydrolysate prepared with an E/S of 47.5 LAPU/g protein for 0.7 h (DH: 24.63%). Further increase in DH value decreased the OH· scavenging ability of the resulting hydrolysate. It can be assumed that further breakdown of proteins into shorter peptides may have caused the reduction of this activity. The precursor proteins responsible for generating peptides with highest OH· scavenging activity could be the protein around 43–55 kDa and the protein around 6.5–11.4 kDa, as the intensity of these bands were reduced at 24.63% DH (Fig. 1). The IC_{50} value of the three hydrolysates possessing highest OH· scavenging activity ranged from 1.56 to 3.06 mg/ml (Table 2). The OH· scavenging activity of unhydrolysed protein was negligible while carnosine (0.5 mg/ml), a dipeptide with proven antioxidant activity [35] showed 63.5% activity under the defined reaction conditions. The OH· scavenging activity was greater in hydrolysates possessing lower DH% and higher PCL compared to those with higher DH% and lower PCL (Fig. 1, Table 1). This suggests the possibility that the relatively long chain peptides acted as potent OH· scavengers. Long chain antioxidant peptides (5–16 amino acid residues) have been also isolated from hydrolysed soy protein [36].

The OH· is the most reactive oxygen radical and its uncontrolled production due to oxidation reactions in human body can severely damage adjacent biomolecules leading to onset of many diseases such as cancer and atherosclerosis [37]. Food-derived antioxidant peptides that provide protection against oxidative stress have been reported. The amino acid sequence of a peptide is important for its antioxidative activity and proper positioning of Glu, Leu and His have been reported to improve radical scavenging activities in antioxidative peptide sequences [38]. However, the research published on OH· scavenging activity of plant protein hydrolysates is scarce.

Bile Acid Binding Ability

The bile acid binding ability of flaxseed protein hydrolysates and unhydrolysed protein was determined in vitro using five individual bile acids. In contrast to other bioactivities assayed, the hydrolysed flaxseed proteins (0.67 mg/ml) demonstrated 0 or very low bile acid binding ability (results are not shown). Therefore the IC_{50} value

was not determined for bile acid binding. Interestingly, the unhydrolysed flaxseed protein (0.67 mg/ml) bound all five bile acids at; 15.4, 17.3, 59.7, 37.9 and 32.2% for SCDC, SC, STC, SGC and SDC, respectively. The flaxseed protein isolate maintained at 50 °C without Flavourzyme treatment (no hydrolysis occurred, DH: 0%) possessed bile acid binding abilities, which were similar or greater than that of the hydrolysed protein (results are not shown). Therefore, the bile acid binding ability observed in the protein hydrolysates, even in very minute amounts, could not be attributed to Flavourzyme catalysed hydrolysis. The ability of cholestyramine, the bile acid binding and cholesterol lowering drug, to bind the individual bile acids was also tested. Cholestyramine (0.67 mg/ml) bound 71.4% SCDC, 27.1% SC, 58.6% STC, 56.9% SGC and 79.3% of SDC. The values for bile acid binding ability of cholestyramine observed in the present study deviated from the previously reported values [9] and this could be attributed to the concentration of cholestyramine and the differences in the assay kit used.

Only a few studies have reported bile acid binding of proteins and their hydrolysates in-vitro, including soy protein, wheat gluten [39] lupin protein [9] and rapeseed protein. In a previous study, lupin protein isolate (1 mg/ml, protein content 91.44%) was able to bind 55.8% SCDC, 54.4% SC, 40.9% STC, 63.9% SGC and 58.4% SDC. The bile acid binding ability shown by flaxseed protein is lower than that of lupin protein isolate except for STC binding [9]. Yoshie-Stark et al. [40] reported that rapeseed protein isolate (1 mg/ml, protein content 70.8%) had a 5.77% SC binding and 10% SDC binding activity, which is much lower than the values shown by flaxseed protein in the present study. The bile acid binding ability of flaxseed protein showed a reduction upon Flavourzyme catalysed hydrolysis. In this study, majority of hydrolysates possessed short PCL due to extensive hydrolysis of flaxseed protein by Flavourzyme. The inability of peptides with short PCL to bind bile acids may be the reason for reduction of the bile acid binding ability upon hydrolysis.

The results of the present study indicated that extensive hydrolysis of flaxseed protein (up to a DH of 70%) could be achieved by Flavourzyme catalysis. Based on the present in-vitro studies, the unfractionated crude flaxseed protein hydrolysates prepared by Flavourzyme catalysed hydrolysis demonstrated ACEI activity and OH· scavenging activity, which indicate their potential to act as antihypertensive and antioxidant agents. The results revealed the impact of DH and PCL on the bioactivities of the hydrolysates. The unhydrolysed protein had better ability to bind bile acids in vitro than hydrolysates.

The physicochemical functionalities of flaxseed protein isolates have been studied and well described, but no significant biological function has been identified. Present findings provide information on extending the potential of flaxseed proteins in regulating elevated blood pressure, OH· mediated reactions and cholesterol lowering ability. The first two aforementioned activities could be obtained by controlled hydrolytic modification of flaxseed protein, while the latter activity was manifested by intact proteins.

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