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Purification of angiotensin I-converting enzyme-inhibitory peptides from the enzymatic hydrolysate of defatted canola meal

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ABSTRACT

Defatted canola meals from seeds of different processing origins were hydrolyzed by Alcalase to give hydrolysates that inhibited angiotensin converting enzyme (ACE) activity. Heat treated meals yielded protein hydrolysates with 50% ACE-inhibitory concentrations of 27.1 and 28.6 μg protein/ml compared with 35.7 and 44.3 μg protein/ml for the none-heat treated meals. Separation of the hydrolysate on a Sephadex G-15 gel permeation column (GPC) yielded a fraction with an IC_{50} value of 2.3 μg protein/ml. Amino acid analysis showed that the GPC fraction contained 45% content of aromatic amino acids in comparison to 8.5% of the hydrolysate. Two peptides, Val-Ser-Val (IC_{50} = 0.15 μM) and Phe-Leu (IC_{50} = 1.33 μM) were purified, and located in the primary structure of canola napin and cruciferin native proteins. The results suggest that canola protein hydrolysate is a potential ingredient for the formulation of hypotensive functional foods.

1. Introduction

As a major contributor to Canada's agri-food industry, Canola, a *Brassica* oilseed, is an internationally registered trademark of the Canola Council of Canada accounting for an annual economic value of approximately Can $11 billion (Canola Council of Canada, 2006; Sosulski, 1983). Canola oil is recognized as a high quality and healthy edible oil, or as a potential source for manufacturing a wide variety of environmentally-friendly products such as biodiesel and bioplastics; the residual canola meal after oil extraction usually contains 35–40% protein content and is mostly utilized as an animal feed or a fertilizer. It has previously been reported that rapeseed proteins contain excellent contents of essential amino acids (Ohlson & Anjou, 1979). Continuous efforts are being applied to improve the methods of protein extraction (Tzeng, Diosady, & Rubin, 1988), or to develop protein hydrolysate for functionality and nutritional improvement (Cumby, Zhong, Naczk, & Shahidi, 2008; Kim, Kim, Lee, & Kang, 1992; Vioque, Sanchez-Vioque, Clemente, Pedroche, & Millan, 2000; Vioque et al., 1999; Yoshie-Stark, Wada, & Wäsche, 2008). However, information on the structure and functionality of bioactive peptides from canola proteins is limited.

In addition to nutritional and functional properties, there is accumulated information in the literature regarding food proteins and health links, which is due in part to the presence of many peptide sequences that are sequestered (and remain inactive) within the primary structure of proteins. These bioactive peptide sequences can be released by careful manipulation of protease activities, *in vitro* and *in vivo*, with potential benefits to human health. Among bioactive peptides, angiotensin converting enzyme (ACE)-inhibitory peptides have been studied extensively in the past two decades. ACE converts angiotensin I to the potent vasoconstrictor, an octapeptide called angiotensin II; ACE also hydrolyzes bradykinin, a vasodilatory agent (Yang, Erdos, & Levin, 1970). Therefore, excessive levels of ACE can lead to increased vasoconstriction and consequently, the development of high blood pressure and associated pathological symptoms. Inhibition of ACE activity by synthesized chemical compounds (drugs) has been shown to lower blood pressure and has been used as a tool in the clinical management of hypertension (Atkinson & Robertson, 1979). The development of food protein-derived ACE-inhibitory peptides is thought to be a safer alternative to commercial ACE-inhibitory drugs. Though effective at lower concentrations when compared to food protein-derived ACE-inhibitory peptides, synthetic hypotensive drugs are reported to have side effects such as dry cough, taste disturbances and skin rashes, as well as alterations in serum lipid metabolism (Atkinson & Robertson, 1979; Israaili & Hall, 1992; Seseko & Kaneko, 1985). Since hypertensive patients often need life-long medications, there is an increasing interest in exploring an alternative

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approach to hypertension intervention such as the use of naturally-occurring ACE inhibitors that will avoid undesirable side-effects associated with ACE-inhibitory drugs.

ACE-inhibitory peptides have been isolated from several food proteins such as milk (Meisel, 2004), fermented milk (Nakamura et al., 1995), alfalfa (Kapel, Rahhou, Lecouturier, Guillochon, & Dhuslster, 2006), soybean (Mallikarjun Gouda, Gowda, Appu Rao, & Prakash, 2006; Wu & Ding, 2002), corn (Suh, Whang, Kim, Bae, & Noh, 2003), bovine skin gelatin (Kim, Byun, Park, & Shahidi, 2001), mung bean (Li, Wan, Le, & Shi, 2006), buckwheat (Li, Matsui, Matsumoto, Yamasaki, & Kawasaki, 2002), rapeseed (Marczak et al., 2003), etc. However, reports on ACE-inhibitory bioactive peptide sequences in canola proteins are scanty. The primary objective of this study was to prepare a peptide fraction from canola proteins with potent ACE-inhibitory activity that can be formulated as a functional food ingredient. The secondary objective involved purification of the primary structures of the most active peptides present in the canola peptide fraction.

2. Materials and methods

2.1. Materials

Two batches of heat-treated commercially defatted canola meals were obtained from Canbra Foods Inc., (Lethbridge, AB, Canada). A none-heat-treated defatted canola meal was obtained from the Department of Animal and Poultry Science, University of Saskatchewan, while a second none-heat-treated meal from dehulled seeds was prepared at Agriculture and Agri-Food Canada Research Centre, Saskatoon, SK, Canada. Alcalase 2.4 L FG was a gift from Novozymes (Franklinton, NC, USA). Pepsin from porcine stomach mucosa, pancreatin, ACE (from rabbit lung), hippuryl-histidyl-leucine (HHL), and captopril were purchased from Sigma Chemicals (St. Louis, MO, USA). HPLC-grade acetonitrile was obtained from Fisher Scientific (Ottawa, ON, Canada). Hippuric acid and trifluoroacetic acid (TFA) were obtained from Acros Organics (Morris Plains, NJ, USA).

2.2. Preparation of canola meal enzymatic hydrolysate

Proximate composition of the meals was determined according to the AOAC (1995) methods. Enzymatic protein hydrolysis of the meal was performed in a temperature- and pH-controlled 1 L reaction vessel equipped with a stirrer. Ground canola meal was mixed with distilled water thoroughly using a magnetic stirrer into 5% ($N \times 6.25$) protein slurry. After adjusting the slurry to pH 8.5 with 2 M NaOH solution and to 60 °C, Alcalase (4%, w/w, on the basis of protein content of slurry) was added to initiate protein digestion. At pre-determined intervals, aliquots of the reaction mixtures were taken, freeze-dried and used for ACE inhibition assay. Temperature and pH values were kept constant during protein digestion and at the end of reaction the enzyme was inactivated by heating the slurry at 85 °C for 15 min. Unhydrolyzed proteins and large peptides as well as other polymers were further precipitated by adjusting the reaction mixture to pH 4.0 and removed by centrifugation at 10,000 × g for 25 min. The clear supernatant was used as the source of peptides for determination of ACE inhibitory activity. A control 'digest' was also prepared as described above but without the addition of alcalase. Supernatant solutions were then freeze dried and stored at −20 °C until further analysis within a month.

2.3. Degree of hydrolysis (DH)

DH of the protein hydrolysates was determined by the trinitrobenzenesulphonic acid method using leucine as the standard (Alder-Nissen, 1979).

2.4. ACE inhibitory determination

The ACE-inhibitory activity was determined using the direct HPLC injection method as reported (Wu, Aluko, & Muir, 2002). Briefly, a total volume of 70 μl (all prepared with 100 mM borate buffer, containing 300 mM sodium chloride, pH 8.3) consisting of 50 μl of 2.17 mM HHL, 2 mU of ACE and a solution of the freeze-dried protein hydrolysate (10 μl) were used. After a 10 min pre-incubation of HHL and food protein hydrolysate, the enzyme was added to initiate the 30 min fixed reaction at 37 °C using an Eppendorf Thermomixer R (Brinkmann Instruments, Inc. New York, NY, USA) at 450 rpm. The reaction was terminated by the addition of 85 μl of 1 M HCl, and 10 μl of this solution was injected (after filtration through 0.2 micron membrane disc) into a C_{18} reverse-phase column (Waters Inc.; Milford, MA) for HA separation and quantification. The operation was performed on Waters™ 2690 Separation Module System (Water Inc.; Milford, MA) controlled by Waters Millennium Chromatography Manager software version 2.15. UV absorbance peak area was integrated automatically with the supplied software at 228 nm. The column was eluted with a two solvent system: (A) 0.05% trifluoroacetic acid (TFA) in water and (B) 0.05% TFA in acetonitrile, with a 5–60.0% B gradient in the first 10 min, maintained for 2 min at 60.0% B, then recycled to 5% B in 1 min and held for 4 min at a flow rate of 0.5 ml/min. External HA standard samples were prepared fresh daily and used for calculation of HA formed in test reactions. The IC_{50} value (concentration of inhibitory substance that reduced ACE activity by 50%) was calculated using a non-linear regression from a plot of activity versus inhibitor concentration of at least five separate determinations.

2.5. Stability of canola ACE-inhibitory peptides against ACE and gastric enzymes

Hydrolysate solution (1.0 mg/ml) was mixed with 0.2 ml of 100 mU/ml ACE solution and incubated for 4 h at 37 °C in Eppendorf Thermomixer R at 450 rpm, after which the reaction mixture was boiled for 15 min to inactivate ACE. IC_{50} value was then determined for the ACE-treated sample. Effect of gastric enzymes was determined by incubation of a 1.6 mg/ml sample first with 2 ml pepsin solution (0.2 mg pepsin/ml of 0.1 M KCl-HCl buffer, pH 2.0) for 4 h at 37 °C in a shaking water bath using 15 ml disposable plastic centrifuge tubes. After neutralizing with 1.0 ml of 2.0 M NaOH, 1.0 ml pancreatin solution (4.47 mg pancreatin/ml of 0.2 M MOPS buffer, pH 8.0) was added to the reaction mixture and incubated for an additional 20 h at 37 °C. The reaction was stopped by boiling for 15 min to inactivate the enzymes followed by IC_{50} determination as described above.

2.6. Gel-Permeation chromatography (GPC)

Preparative GPC separation of canola hydrolysate was performed on XK 50/60 (Amersham GE, Montreal, PQ) column packed with a Sephadex G-15™ matrix. The column was coupled to an ÄKTA explorer 10XT system and equilibrated with 20% (v/v) aqueous ethanol solution at a flow rate of 2.0 ml/min. Sample was injected, eluted at 2.0 ml/min and detected at 214 nm. The molecular weight distribution of canola meal hydrolysates/peptides was examined by GPC on the same ÄKTA system but with a Superdex Peptide 10/30 column (Amersham GE, Montreal, PQ). A 25 μl aliquot of 5 mg/ml canola meal hydrolysate solution (prepared with 50% aqueous acetonitrile solution) was injected directly into the Superdex Peptide column (Amersham GE, Montreal, PQ). Elution was performed isocratically with the aqueous acetonitrile solution that contained 0.05% trifluoroacetic acid (TFA) at a flow rate of 0.3 ml/min; eluted peptides were detected at 214 nm. All

standard peptide markers were purchased from Sigma Chemicals (St. Louis, MO): cytochrome C, molecular weight (MW) of 12500 Da, gastrin I (MW = 2126 Da), bacitracin (MW = 1450 Da), substance P (MW = 1348 Da), GGYR (MW 451.5), (poly-Glycine) (MW, 360).

2.7. Peptide purification and identification

Active fractions from the preparative GPC was dissolved in 1% acetic acid water (v/v) solution at a concentration of 100 mg/ml for preparative HPLC chromatography. A segmented column composed of three PrepPak 40 mm Cartridges (Bondapack C_{18}, 15–20 μm, 40 × 100 mm, Waters Inc, Milford, MA. USA) and a guard column (40 × 10 mm, Waters Inc, Milford, MA. USA) was coupled with an HPLC 4000 system (Waters Inc, Milford, MA. USA). Instrument control, data collection and analysis were undertaken using Millennium Chromatography Manager software v 2.15. The sample (20 ml) was automatically injected via a solvent delivery system. The column was initially eluted with 100% solvent A (water containing 1% acetic acid) for 10 min, and then with a linear gradient of solvent B (methanol) in solvent A up to a concentration of 45% B within 178 min after which elution reverted back to 100% A within 10 min. The flow rate was 50 ml/min. The absorbance profile was monitored at 214 nm; peak fractions were collected, concentrated under reduced pressure, and freeze-dried. The most active fraction from the preparative HPLC was further purified by Sephasil™ Peptide C_{18} ST 10/250 column (Amersham GE, Montreal, PQ) using the ÄKTA explorer 10XT. The column was eluted at a flow rate of 5 ml/min by a two solvent system: A, 20 mM sodium phosphate buffer containing 0.05% TFA and B, 20 mM sodium phosphate prepared in 80% acetonitrile that contained 0.05% TFA. After 5 column volumes (CV) of elution at 5% B, the gradient was increased to 20% B within 15 CV. The injection volume was 500 μl of 200 mg/ml solution of the most active preparative chromatography fraction. The absorbance was monitored at 214 nm and fractions were collected at 3 ml per tube. Active fractions were re-chromatographed twice on the AKTA system first with the same Sephasil™ Peptide C_{18} ST 10/250 column and subsequently with a Sephasil Peptide C_{18} ST 4.6/250 column to produce the final purified peptides. The first re-chromatography involved solvent system A (water containing 0.1% TFA) and B (acetonitrile containing 0.1% TFA) at the gradient of 10% B to 30% B in 7 CV at the flow rate of 5 ml/min. The second and final purification step involved the same solvents A and B but at a gradient of 5% B to 40% B in 10 CV using a flow rate of 1.5 ml/min. The injection volume was 30 μl of 25 mg/ml sample solution and elution was monitored at 214 nm.

Identity of the peptides in the purified peaks from the ST 4.6/250 column were determined by atmospheric pressure chemical ionization (APCI) mass spectrometry using a Quattro LC liquid chromatograph/Mass Spectrometer (Micromass UK Ltd., Wythenshawe, Manchester, UK) equipped with APCI probe, Z-Spray interface, separation module and photodiode array detector (Waters Inc. Milford, MA, USA). Instrument control and data analysis were performed using the MassLynx software (Micromass UK Ltd., Wythenshawe, Manchester, UK). A peptide Sample (10 μl) was chromatographed on a Symmetry C_{18} column (2.0 × 150 mm; 5 μm; Waters Inc. Milford, MA, USA). The sample was eluted (0.3 ml/min) with a two solvent system: (A) 0.1% formic acid (FA) in water and (B) 0.1% FA in acetonitrile, with a 5–60% acetonitrile gradient for the first 10 min, maintained for 2 min at 60% acetonitrile, then returned to 5% acetonitrile for 1 min. This was followed by isocratic elution for 4 min at the constant flow rate of 0.3 ml/min. Positive and negative ion intensities were recorded from 50 to 500 m/z with a 1.5 s scan time. The analyzer vacuum was $2.2e^{-5}$ torr.

2.8. Amino acid analysis

Amino acid analyses were carried out using an HPLC system after the samples were hydrolyzed with 6 M HCl according to the method of Bidlingmeyer, Cohen, and Tarvin (1984). Performic acid oxidation of samples was used for determining cysteine and methionine (Gehrke, Wall, Absheer, Kaiser, & Zumwalt, 1985)

Table 1
Proximate composition of four canola meals and angiotensin converting enzyme (ACE)-inhibitory activities of their protein hydrolysates

Meal type	Moisture (%)	Oil (%)	Ash (%)	Protein (%)	IC_{50} of hydrolysate (μg protein/ml)[a]
None-heat treated (from undehulled seeds)	7.04	3.26	5.99	55.24	35.7
None-heat treated (from dehulled seeds)	6.65	2.72	7.31	49.58	44.3
Heat-treated batch 1[b]	11.36	1.72	7.81	36.78	27.1
Heat-treated batch 2[b]	12.25	2.90	7.47	39.42	28.6

[a] Concentration of protein hydrolysate that reduced ACE activity by 50%.
[b] Commercial meals from undehulled seeds.

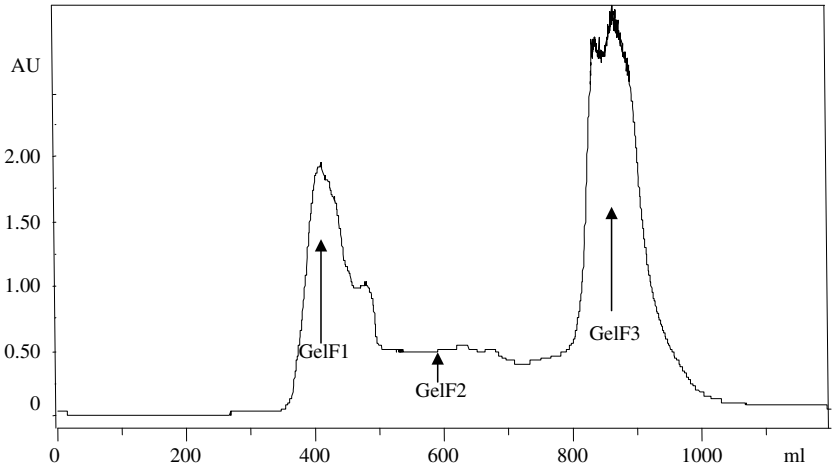


Fig. 1. Sephadex G-15 chromatograph of canola meal protein hydrolysate in XK 50/60 column coupled with AKTA system under isocractic condition eluted with 20% aqueous ethanol solution.

while alkaline hydrolysis was used to prepare samples for tryptophan analysis (Landry & Delhaye, 1992).

2.9. Statistical analysis

Duplicate determinations were made and the data were analyzed using a one-way analysis of variance (ANOVA) using SAS, version 9.0; significant differences were accepted at $p<0.05$.

3. Results and discussion

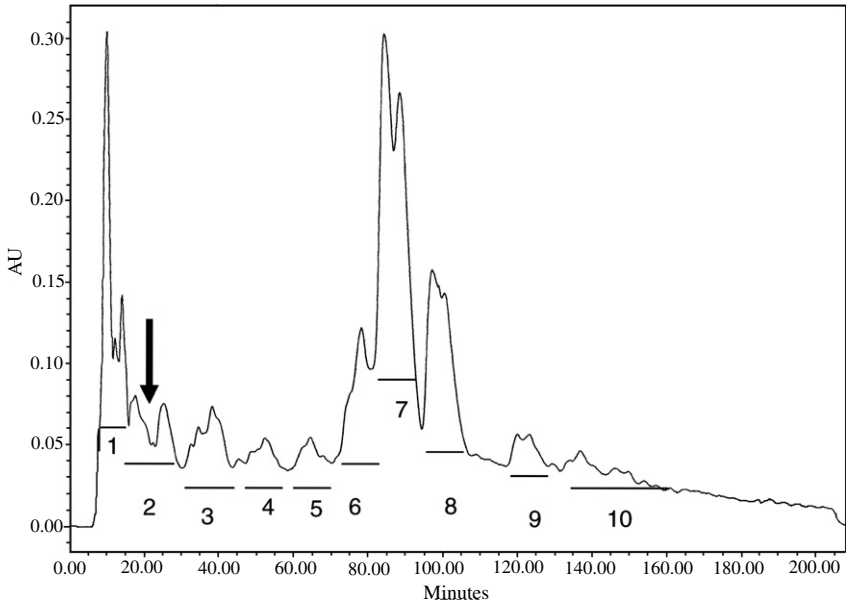
3.1. Effect of canola meal type on the ACE-inhibitory activity of protein hydrolysates

Industrially defatted canola meal contains high insoluble protein content, caused by high temperature applied in the oil extraction process, which greatly limits its application in food products. Unlike other oilseed meals such as soybean, peanut, and sunflower seed, canola meal shows low nitrogen extractability in alkali solutions over a broad pH range. This can be ascribed to the fact that canola proteins have a very complicated composition and contain

Table 2
Amino acid composition (%) of canola meal, protein hydrolysate and most active gel filtration fraction

Amino acid	Canola meal	Hydrolysate	GelF3
Asx[a]	7.43	8.33	8.04
Glx[b]	15.51	19.52	10.04
Ser	4.51	4.30	3.60
Gly	4.56	5.03	5.40
His	2.14	2.06	1.20
Arg	6.78	6.86	2.43
Thr	4.46	3.96	2.31
Ala	3.99	4.50	2.73
Pro	6.48	6.05	2.49
Tyr	2.64	2.91	19.95
Val	4.94	5.05	2.88
Met	2.04	1.76	1.38
Cys	1.82	1.82	1.71
Ile	3.77	3.70	5.04
Leu	6.61	6.56	5.28
Phe	3.67	3.55	17.25
Lys	4.71	5.27	0.84
Trp	1.42	1.96	8.25

[a] Aspartic acid + asparagine.
[b] Glutamic acid + glutamine.



ACE inhibitory activity of fractions

Number of fraction	ACE inhibitory activity (%)
Delta-1	40.6
Delta-2	81.3
Delta-3	48.8
Delta-4	25.9
Delta-5	31.0
Delta-6	35.2
Delta-7	27.1
Delta-8	41.5
Delta-9	23.9
Delta-10	21.8

Fig 2. Preparative reverse-phase HPLC chromatogram of the most active fraction from gel permeation chromatography. Fraction 2, which had the highest ACE-inhibitory activity was pooled and subjected to FPLC fractionation and purification protocols. The inserted table showed the ACE inhibitory activity of each fraction.

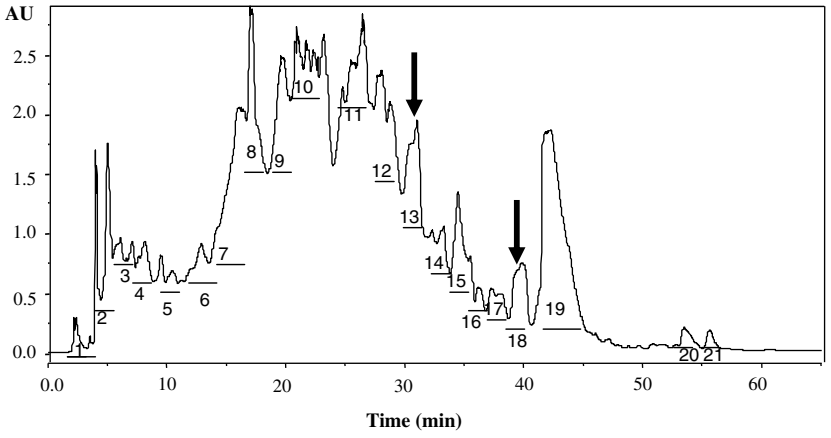
proteins that differ widely in their isoelectric points (pH 4–11) and molecular weights (13–320 kDa) (Klockeman, Toledo, & Sims, 1997; Lönnerdal, Gillberg, & Törnell, 1977). Consequently, preparation of canola proteins usually results in lower yield compared with other oilseed meal proteins. Therefore, instead of using canola protein isolate, we utilized canola meal as raw material for production of soluble protein hydrolysates in order to increase soluble peptide yield and simplify the process.

Table 1 shows the result of the proximate composition of four types of defatted canola meals as well as the ACE-inhibitory IC_{50} values of the corresponding enzymatic protein hydrolysates. The IC_{50} values of the hydrolysates were in the range of 27.1–44.3 μg protein/ml; heat-treated meal hydrolysates had a higher ACE-inhibitory activity than the none-heat treated meal hydrolysates (Table 1). It is known that proteins in heat-treated defatted meal are mostly denatured due to the high temperatures applied during oil extraction. Thus, the heat-treatment probably increased susceptibility to protease digestion and the release of more active ACE-inhibitory peptides. Previous reports on the IC_{50} values from other food protein Alcalase hydrolysates were in the range of 180–2530 μg/ml (Sugiyama et al., 1991; Matsui et al., 1993; Hyun & Shin, 2000), which are higher than those obtained in the present work.

ACE-inhibitory activity and DH values increased in parallel with prolonged incubation of canola meal with Alcalase (*data not shown*). No ACE-inhibitory activity was detected before hydrolysis. The highest ACE-inhibitory activity was reached after 120 min of reaction, corresponding to a DH value of 20.7%; further hydrolysis did not produce additional increase in inhibitory activity. Our results showed that protein hydrolysis was necessary for the release of ACE-inhibitory peptides from an inactive form within the sequence of the native canola proteins. In comparison, higher levels of proteolysis were required to produce very active enzymatic digests from chickpea protein isolate and legumin, with DH values of 30% (Pedroche et al., 2002) and 27% (Yust et al., 2003), respectively.

3.2. Simulated gastrointestinal tract stability of canola protein hydrolysate

Previous reports have suggested that some ACE-inhibitory peptides failed to show potent hypotensive activity *in vivo* although they exhibited powerful ACE-inhibitory activity *in vitro* (Fujita,



ACE inhibitory activity of fractions

Number of fraction	ACE inhibitory activity (%)
PRC-1	1.4
PRC-2	1.5
PRC-3	ND[a]
PRC-4	ND
PRC-5	ND
PRC-6	ND
PRC-7	11.0
PRC-8	7.1
PRC-9	ND
PRC-10	3.0
PRC-11	8.5
PRC-12	23.5
PRC-13	64.2
PRC-14	11.9
PRC-15	6.0
PRC-16	3.4
PRC-17	7.6
PRC-18	38.2
PRC-19	6.2
PRC-20	8.4
PRC-21	ND

[a]: not ACE inhibitory activity was detected.

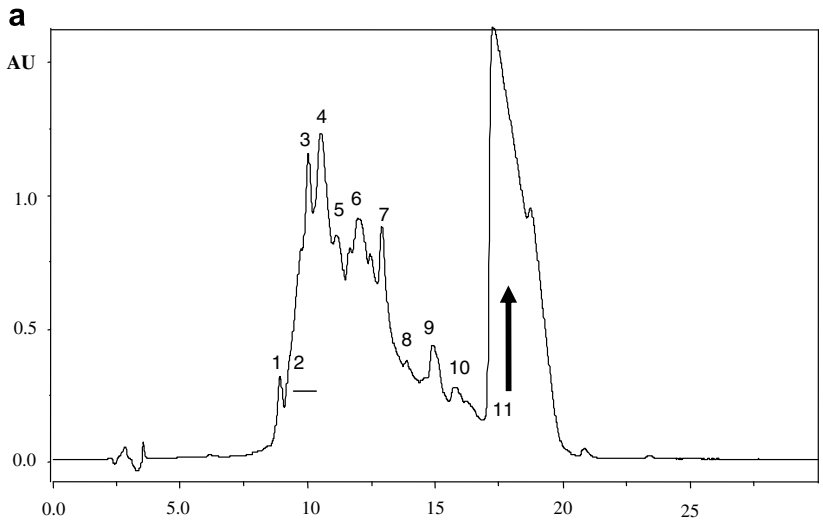
Fig. 3. First semi-preprative reverse-phase FPLC chromatogram of the most active fraction from the preparative HPLC step. Fractions 13 and 18, which had the highest ACE-inhibitory activity were pooled and subjected to a final FPLC purification. The inserted table showed the ACE inhibitory activity of each fraction.

Yokoyama, & Yoshikawa, 2000). There are two possibilities, one is that the peptides may be susceptible to hydrolysis by ACE under physiological conditions; two, the peptides may be destroyed by gastric enzymes. Therefore, prior to further processing, it is necessary to determine the susceptibility of the active protein hydrolysate to simulate gastric digestion. IC_{50} values of the true peptide inhibitors are not substantially altered after incubation with ACE, while inhibitory activities of the peptide substrates are substantially reduced by incubation with ACE. Simulated gastrointestinal tract stability studies showed that the ACE-inhibitory activity of the most active canola meal protein hydrolysate was not altered after incubation with ACE (25.7 μg protein/ml compared with 27.1 μg protein/ml of the control protein hydrolysate). Furthermore, the IC_{50} value of the hydrolysate was decreased after incubation with gastric enzymes (18.6 μg protein/ml), probably due to the generation of more active peptides through the action of GIT enzymes. Our results suggest that peptides in the canola protein hydrolysates are resistant to ACE and gastric enzymes, which means they could potentially exert a desirable physiological effect such as blood pressure reduction.

3.3. GPC fractionation

The use of Sephadex G-15 chromatography led to a fraction with greatly improved ACE-inhibitory activity (Fig. 1). It was observed that the ACE-inhibitory activity of each fraction showed direct relationship with that of the molecular size. GelF3 (smallest peptide size) had the highest ACE-inhibitory activity (IC_{50} = 2.3 μg protein/ml) while GelF1 (biggest peptide size) had an IC_{50} value of 117.1 μg protein/ml; GelF2 had IC_{50} value of 21.4 μg protein/ml. The IC_{50} value obtained for GelF3 is almost 35-fold lower than that the most ACE-inhibitory fraction (IC_{50} = 80 μg/ml) obtained from a Sephadex G-25 gel chromatography of albumin hydrolysate (Hyun et al., 2000). The IC_{50} value obtained for GelF3 in this work is superior to the 29 μg protein/ml from a thermolysin hydrolysate of dried bonito (Yokoyama, Chiba, & Yoshikawa, 1992) and the 15 μg protein/ml value obtained for a 10% ethanol-eluted fraction of the sardine protein hydrolysate (Matsui et al., 1993). To the best of our knowledge, the bonito hydrolysate fraction is the most potent ACE-inhibitory peptides reported for a food protein in literature and has been confirmed with demonstrated *in vivo* hypotensive activity; the hydrolysate has been approved as part of the "Foods for Specified Health Use" in Japan (Fujita, Yasumoto, Hasegawa, & Ohshima, 2001; Yokoyama et al., 1992; Fujita, Yokoyama, Yasumoto, & Yoshikawa, 1995; Fujita et al., 2000; Sugiyama et al., 1991). Therefore, the ACE-inhibitory activity of the GelF3 fraction compares favorably with that of the Japanese-approved bonito hydrolysate. However, it should be realized that gel filtration chromatography may not be feasible for large-scale production of bioactive peptides.

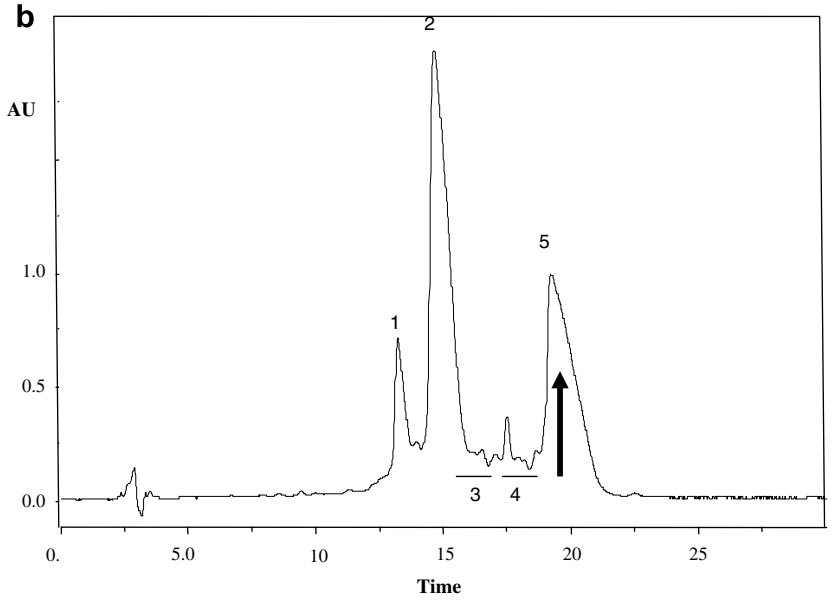
Overall, the peptide size of the GelF3 fraction (77% content of peptides with <870 Da) was smaller than that of the original canola



ACE inhibitory activity of fractions

Number of fraction	ACE inhibitory activity (%)
F13-1	15.1
F13-2	11.0
F13-3	6.1
F13-4	9.2
F13-5	19.7
F13-6	6.2
F13-7	19.3
F13-8	1.4
F13-9	5.2
F13-10	15.9
F13-11	42.4

Fig. 4. Second semi-preparative reverse-phase FPLC chromatogram of the most active fractions from the first semi-preparative FPLC step. a: fraction 13 and b: fraction 18 from Fig. 3. Fraction 11 in 'a' and fraction 5 in 'b' produced single peaks on a reverse-phase HPLC analytical column and were subjected to amino acid sequencing. The inserted table showed the ACE inhibitory activity of each fraction.



ACE inhibitory activity of fractions

Number of fraction	ACE inhibitory activity (%)
F8-1	4.2
F8-2	ND[a]
F8-3	ND
F8-4	39
F8-5	84

[a]: not ACE inhibitory activity was detected.

Fig. 4 (*continued*)

protein hydrolysate (60% content of peptides with <870 Da). Peptide size is an important property of bioactive protein hydrolysates because it has been reported that small peptides especially di- and tripeptides could be absorbed more efficiently with greater efficiency than free amino acids and bigger peptide molecules (Matthews, 1977). Thus, it is possible that the GelF3 fraction peptides might be more readily assimilated from the GIT when compared to the whole canola protein hydrolysate. The amino acid composition of canola meal and canola meal protein hydrolysate was quite similar but was different from that of the GelF3 fraction (Table 2). The most noticeable difference was in the contents of aromatic amino acids (Tyr, Phe, Trp), which were substantially increased with a total of approx. 8.5% in the hydrolysate compared with 45.2% in the GelF3 fraction. Previous reports have suggested that the presence of hydrophobic (aromatic or branched chain) amino acids may contribute to increased activities of ACE-inhibitory peptides (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980; Wu, Aluko, & Nakai, 2006). Therefore, it is possible that the potency of the GelF3 against ACE may be due largely to the very high content of aromatic amino acid-containing peptides. It has also been suggested that the presence of proline residues contributes to the potency of most naturally-occurring ACE-inhibitory peptides (Cheung et al., 1980). However, our results provide a different argument since the GelF3 fraction contained lower amounts of proline than the protein hydrolysate. Table 2 shows that a reduction in the contents of positively charged amino acids may have contributed to the high ACE-inhibitory activity of GelF3 (His + Arg + Lys = ~4.5%) when compared to the canola protein hydrolysate with approx. 14.2% content of the basic amino acids.

3.4. Peptide purification and amino acid sequence

Fig. 2 shows the preparative HPLC chromatogram of the canola peptide fraction, GelF3 from GPC. From 10 collected fractions, fraction 2 (marked with arrow) showed the highest ACE-inhibitory activity. Fraction 2 was further purified on a semi-preparative HPLC as shown in Fig. 3. From 21 collected fractions, F13 and F18 had the highest ACE-inhibitory activities. F13 and F18 were further purified on the same column using a different solvent system. Peaks with the highest ACE inhibitory activities were F13-11 (Fig. 4a) and F18-5 (Fig. 4b) and were determined to be pure because each appeared as a single peak on an analytical column (*data not shown*). Amino acid sequences were identified using HPLC-MS/MS and shown to be Val-Ser-Val for F13–11 and Phe-Leu for F18-5 with ACE-inhibitory IC_{50} values of 0.15 μM and 1.33 μM, respectively. Both sequences exist in napin and cruciferin, two major canola protein components. Sequence of Val-Ser-Val can be found in the primary structure of napin 2 precursor (f154–156) and cruciferin BNC1 precursor (f199–f201). Sequence of Phe-Leu can also be found in the primary structure of napin 2 precursor (f6–7 and f16–17) and cruciferin BNC1 precursor (f7–8 and f344–345). The IC_{50} values were favorably comparable with previously reported ACE inhibitory peptides from rapeseed subtilisin hydrolysate for Ile-Tyr (3.7 μM), Arg-Ile-Tyr (28 μM), Val-Trp (1.6 μM) and Val-Trp-Ile-Ser (30 μM) (Marczak et al., 2003). It is very likely that subtilisin Carlsberg (type VIII from *Bacillus licheniformis*) used by Marczak et al. (2003) is similar to the enzyme we applied in our study. Our results showed that differences in substrate preparation and method purification could result in different peptide sequences. Phe-Leu

has previously been reported in Alcalase-hydrolyzed whey protein with an IC_{50} value of 16 μM (Eto, Ito, & Nishioka 1998). However, to the best of our knowledge, the present work is the first to report the purification of ACE-inhibitory peptide of Val-Ser-Val from a food protein hydrolysate. Val-Ser-Val and Phe-Leu had greater ACE-inhibitory potencies when compared with other ACE-inhibitory peptides such as Lys-Asp-Tyr-Arg-Leu, Val-Thr-Pro-Ala-Leu-Arg, and Lys-Leu-Pro-Ala-Gly-Thr-Leu-Phe with ACE-inhibitory IC_{50} values of 26.5, 82.4, and 13.4 μM, respectively as previously reported by Li et al. (2006). The IC_{50} value of Val-Ser-Val is lower than those of Gly-Pro-Leu (2.55 μM) and Gly-Pro-Val (4.67 μM) that were isolated from bovine skin gelatin (Kim et al., 2001), Leu-Arg-Ile-Pro-Val-Ala (0.38 μM) and Met-Arg-Trp (0.6 μM) that were purified from spinach rubisco (Yang, Marczak, Yokoo, Usui, & Yoshikawa, 2003). However, Phe-Leu is less active than Leu-Lys-Pro (0.32 μM), Ile-Lys-Trp (0.21 μM), Phe-Lys-Gly-Arg-Tyr-Tyr-Pro (0.55 μM) obtained from thermolysin digest of chicken muscle, as well as Phe-Phe-Gly-Arg-Cys-Val-Ser-Pro (0.4 μM) and Glu-Arg-Lys-Ile-Lys-Val-Tyr-Leu (1.2 μM) from peptic digest of ovalbumin (Fujita et al., 2000).

4. Conclusions

The study confirmed that bioactive peptides can be released from canola proteins by protease-mediated digestion. It was demonstrated that defatted canola meal could be applied directly as raw material for the production of bioactive peptides without the extraction of proteins. By obviating the need for use of the more expensive protein isolate, our canola meal-based method provides a simple way to produce the ACE-inhibitory protein hydrolysate. From amino acid analysis, it was evident that lower contents of basic amino acid residues in combination with high contents of aromatic and hydrophobic (bulky) amino acids are responsible for the ACE-inhibitory potency of canola protein hydrolysate. Though it may not be commercially feasible to use the purified peptides in formulating food products, the more easily prepared hydrolysate fraction seems sufficiently potent against ACE. Moreover, the present results open up opportunities for scientists to find ways of enriching canola protein's primary structure with the identified ACE-inhibitory peptides such that the protein hydrolysate can contain high levels of the desirable peptides. Overall, bioactive efficacy of the hydrolysate needs confirmation using an appropriate animal disease (hypertension) model. The development of bioactive peptides from canola meal may open-up new economic opportunities for increased value-added utilization of canola.

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