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ARTICLE

Angiotensin-I Converting Enzyme Inhibitory Activity of Hydrolysates from Oat (*Avena sativa*) Proteins by *In Silico* and *In Vitro* Analyses

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The potential for producing antihypertensive peptides from oat proteins through enzymatic hydrolysis was assessed *in silico* and confirmed *in vitro*. Thermolysin (EC 3.4.24.27) was predicted using BIOPEP database as the enzyme that would theoretically produce the most angiotensin I converting enzyme (ACE) inhibitory peptides from oat. Experimental evidence confirmed that strong ACE-inhibitory activity was produced under various hydrolysis conditions. Hydrolysates produced under high enzyme-to-substrate ratio (3%) short time (20 min) (HEST) and low enzyme-to-substrate ratio (0.1%) long time (120 min) (LELT) conditions had IC_{50} values of 30 and 50 μg/mL, respectively. After simulated gastrointestinal digestion, the IC_{50} of the HEST hydrolysate was 35 μg/mL whereas the IC_{50} of the LELT hydrolysate was higher at 85 μg/mL. Ultrafiltration revealed that potent ACE-inhibitory peptides had molecular weights below 3 kDa. This study demonstrates the usefulness of *in silico* analysis to select enzymes for hydrolysis of proteins not previously examined as sources of bioactive peptides.

KEYWORDS: Oat protein isolate; in silico analysis; ACE-inhibitory peptides; thermolysin; hydrolysis conditions

INTRODUCTION

Cardiovascular disease is the world's leading cause of death resulting in 17.5 million deaths in year 2005 (*1*). Since hypertension is a major risk factor for cardiovascular disease, the maintenance of healthy blood pressure is considered important. Angiotensin-I converting enzyme (ACE, EC 3.4.15.1) is a multifunctional enzyme in the renin−angiotensin system that plays a key role in regulating blood pressure, fluid and electrolyte balance. ACE catalyzes the conversion of angiotensin I into the vasoconstrictor angiotensin II, and may also catalyze degradation of the vasodilator bradykinin in the kallikrein−kinin system and/or neuropeptides that interact with the cardiovascular system (*2*). Synthetic ACE-inhibitor drugs such as captopril, enalapril and lisinopril effectively lower blood pressure in individuals with hypertension, lowering risk of coronary heart disease and stroke, and improving prognosis of patients with cardiac failure and diabetic nephropathy (*3*). However, these drugs are often accompanied by undesirable side effects, including a persistent nonproductive dry cough that has been reported in up to 44% of patients, as well as less common but more serious side effects such as allergic reactions triggering anaphylaxis, retention of potassium (hyperkalemia) and difficulty swallowing or breathing due to angioedema (*3*).

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In contrast to the synthetic ACE inhibitor drugs, no such side effects have been observed for ACE inhibitors derived from food peptides (*4*,*5*). Most studies for deriving ACE-inhibitory peptides from enzymatic hydrolysis of food proteins have focused on milk proteins (*6*−*10*) and fish proteins (*11*−*13*) while a few have explored the potential of alternatives such as corn (*14*), rapeseed (*15*), soy (*16*) and wakame (*17*, *18*). With the growing demand for natural health products that can help to maintain healthy blood pressure, there is a need to consider other possible sources that can tailor to the wide spectrum of consumers with different dietary restrictions.

Oats (*Avena sativa*) are widely consumed as a source of soluble fiber, and studies have shown the benefits of consumption of dietary fiber, particularly the β-glucan fraction from oats (*19*). Recent studies have demonstrated the beneficial effects of the consumption of oats on lowering blood pressure of patients with hypertension and obesity (*20*, *21*). The proximate composition of oatmeal or rolled oat, which is representative of oat groat, has been reported to contain 8−12% moisture, 11−16% protein, 4−10% fat, <2% ash and 66−73% carbohydrate (*22*). Despite the high contents of protein as well as essential amino acids in oat compared to most cereals (*22*), there seems to be limited recognition of the nutritional properties of oat proteins (*23*). Furthermore, the possibility of generating peptides with ACE-inhibitory activity from oat proteins has not been reported.

The traditional method to screen for ACE inhibitors from a novel substrate involves selecting proteases comprising the ability to liberate potent ACE-inhibitory peptides based on literature reports and testing for the activity experimentally. However, the

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key role of the substrate sequence in affecting the generation of ACE inhibitors could make this approach costly and time-consuming. The availability of BIOPEP, a database of biologically active peptide sequences, allows for theoretical prediction of potential bioactivity of different substrates and their corresponding activity after hydrolysis using enzyme with known cleavage specificities (*24*). Vecchi and Anon (*25*) have recently used the BIOPEP database to successfully identify two ACE-inhibitory tetrapeptides from *Amaranthus hypochondriacus* 11S globulin.

Therefore, the objectives of the present study were (1) to use an *in silico* approach to evaluate the potential of using oats as a protein source for generation of ACE-inhibitory peptides, and to screen for candidate enzymes to hydrolyze the oat protein for this purpose; and (2) to conduct experiments that would verify whether ACE-inhibitory activity could in fact be produced by the action of the selected enzyme on oat protein isolate. The effects of hydrolysis conditions, *in vitro* simulated gastrointestinal digestion, and ultrafiltration on ACE-inhibitory activity of oat protein hydrolysates were also investigated.

MATERIALS AND METHODS

Materials. Organic hulled oat groats were purchased from Galloway's Specialty Foods (Richmond, BC, Canada). Thermolysin from *Bacillus thermoproteolyticus* Rokko (69 units/mg protein), subtilisin A from *Bacillus licheniformis* (type VIII, 8 units/mg solid), trypsin from porcine pancreas (17600 units/mg solid), pepsin A from porcine gastric mucosa (3300 units/mg solid), pancreatin (8 × USP), angiotensin-converting enzyme from rabbit lung (ACE, 1 unit), *N*-hippuryl-his-leu tetrahydrate (HHL) and bovine serum albumin (BSA) were from Sigma-Aldrich Canada (Oakville, ON, Canada). The BCA Protein Assay Reagents A and B were from Thermo Fisher Scientific Inc. (Rockford, IL) while 2,4,6-trinitrobenzenesulfonic acid (TNBS) was from Fluka Biochemika (Oakville, ON, Canada).

BIOPEP Analysis. The prediction of potential antihypertensive peptides from oat proteins was performed using the BIOPEP database available at http://www.uwm.edu.pl/biochemia/index_en.php (*24*). The sequences for oat 11S globulin, 12S globulin and avenin-3 (UniProtKB/TrEMBL ID Q38780, O49257 and P80356, respectively, obtained from the UniProt Knowledgebase of ExPASy Proteomics Server available at http://www.expasy.ch/) (*26*), were investigated using BIOPEP for their "profiles of potential biological activity", in particular for sequences within the oat proteins matching peptide sequences reported in the literature as exhibiting either *in vitro* ACE-inhibitory or *in vivo* antihypertensive activity, both of which have been included under the "antihypertensive activity" category in the BIOPEP database.

Subsequently the oat 11S and 12S globulin and avenin-3 sequences were submitted for analysis using the "enzyme action" tool available in the BIOPEP database to predict the theoretical release of sequences with "antihypertensive activity" by hydrolysis with proteases with known cleavage specificities (*24*). In addition to the action of single enzymes, the combined action of pepsin and trypsin as well as that of pepsin, trypsin and chymotrypsin A, often used to mimic the gastrointestinal digestion process, were also included in the analysis.

Preparation of Oat Protein Isolate (OPI). Organic hulled oat groats were ground with a Galaxie cyclotrol 8 Osterizer (Sunbeam Corporation (Canada) Ltd.; Mississauga, ON, Canada) and sieved through a 1.00 mm mesh before storage at −18 °C. The ground oats were defatted with hexane using a Labconco Goldfish solvent extractor (Labconco Corporation; Kansas City, MO) according to the method of Shahidi (*27*), and the resulting defatted ground oats were used to prepare OPI by the alkaline extraction−isoelectric precipitation method described by Ma (*28*) with the following modifications. The speed of centrifugation in the Du Pont Sorvall Centrifuge RC 5B (Mandel Scientific Co. Ltd.; Guelph, ON, Canada) was 8000*g* (instead of 4000*g*) to ensure successful formation of pellets, and the pellets isolated from the alkaline slurry were re-extracted for more complete extraction of proteins. The extracts were pooled together and lyophilized to produce OPI powder.

Oat Protein Hydrolysis. OPI (3% w/v in distilled, deionized water ddH_2O) was adjusted to the pH that was optimal for each protease using NaOH or HCl and equilibrated to the optimal temperature in a Magni Whirl constant temperature bath with shaker (Blue M Electric Company; Blue Island, IL) before the addition of enzyme(s) (0.033−3% w/w OPI) and incubation for the designated duration time (up to 18 h). The protease treatments used in this study were pepsin (pH 1.3, 37 °C), subtilisin (pH 7.5, 37 °C), thermolysin (pH 8.0, 70 °C) and a sequential pepsin (pH 1.3, 37 °C, first 3 h) + trypsin (pH 7.6, 37 °C, after 3 h) hydrolysis. OPI without addition of protease at zero time of hydrolysis was used as a control. After the designated time of hydrolysis, to an aliquot of each reaction mixture (referred to as oat protein hydrolysate, OPH) was added 24% TCA in a 1:1 (v/v) ratio to terminate the reaction for analysis of degree of hydrolysis, while another aliquot was heated in a boiling water bath for 10 min to inactivate the enzyme before assay of angiotensin I converting enzyme (ACE) inhibitory activity.

Response Surface Methodology for Oat Protein Hydrolysis by Thermolysin. Response surface methodology was conducted using a central composite rotatable design with two factors, namely, the amount of thermolysin (30−900 μg) and the duration of hydrolysis (10−180 min). Of the 13 experiments in the design, there were 9 unique conditions and 5 replicates of the center point generated using the Minitab statistical program version 15.0 (Minitab Inc.; State College, PA). The concentration of OPI was fixed at 3% w/v while the pH and temperature were maintained at 8 and 70 °C, respectively.

***In Vitro* Simulated Gastrointestinal (GI) Digestion of Oat Protein Hydrolysate.** *In vitro* simulated GI digestion with pepsin and pancreatin was performed on 1% w/v hydrolysates in ddH_2O. Hydrolysates produced with 3% thermolysin for 20 min (referred to as high enzyme-to-substrate ratio short time or HEST) and 0.1% thermolysin for 120 min (referred to as low enzyme-to-substrate ratio long time or LELT) were digested according to the protocol of Lo and Li-Chan (*16*) and Cinq-Mars and others (*29*), respectively.

Ultrafiltration of Oat Protein Hydrolysate. HEST and LELT hydrolysates were centrifuged at 1465*g* for 10 min. The supernatant was ultrafiltered sequentially using Ultracel Amicon model 8400 ultrafiltration unit (Millipore Corporation; Bedford, MA) with molecular weight cutoff membranes YM-10 (10 kDa), YM-3 (3 kDa), YM-1 (1 kDa) and YC-05 (0.5 kDa) under maximum pressure of 30 psi nitrogen gas at room temperature. Ultrafiltered fractions were lyophilized and stored at −18 °C until further use.

Degree of Hydrolysis (DH). The degree of hydrolysis was monitored by the content of free amino groups determined in triplicate by the TNBS reaction according to the method described by Lo and Li-Chan (*16*) with the following modifications. OPH was centrifuged at 12100*g* for 7 min (instead of 12350*g* for 5 min) using a VWR Galaxy 16 microcentrifuge (VWR Scientific Products; Bristol, CT), and the supernatant was diluted 20-fold in ddH_2O before the addition of sodium tetraborate buffer and TNBS. DH was expressed as hydrolysis equivalent (h) in leucine amino equivalents by reference to a leucine standard curve.

ACE Inhibitory Activity Assay. ACE-inhibitory activity of OPH was analyzed in triplicate according to the method of Cushman and Cheung (*30*) as adapted by Cinq-Mars and Li-Chan (*12*) with the following modifications. Thirty microliters of OPH was preincubated with 30 μL of ACE (2.5 mU) at 37 °C for 15 min (instead of 1 h), after which 150 μL of 6.5 mM HHL (instead of 7.8 mM) was added. ACE-inhibitory activity was determined by measuring the absorbance at 228 nm produced by liberation of hippuric acid from HHL by the action of ACE in the absence or presence of OPH. The IC_{50} of the samples was determined from cubic regression of the % ACE inhibition values at five or more OPH concentrations in the range of 0.01−0.5 mg/mL, each analyzed in triplicate.

Statistical Analysis. Analysis of Variance General Linear Model (ANOVA-GLM) to analyze significant difference at $P < 0.05$ between samples and quadratic regression of the response surface model were performed using Minitab statistical software version 15. Cubic regression analysis for estimation of IC_{50} of ACE-inhibitory peptides was done using Microsoft Office Excel.

RESULTS AND DISCUSSION

***In Silico* Prediction of Potential ACE-Inhibitory Peptides of Oat Proteins by BIOPEP.** For this study, oat 11S globulin, 12S

Table 1. Summary of Antihypertensive Peptide Sequences Found in Oat Proteins Using Biopep "Profile of Potential Biological Activity" Tool

Protein	Peptide Sequence[a]	Total	Examples of Overlapping Antihypertensive Peptide Sequences[b]
11S Globulin	SF(3), FP(3), LF(3), FY(1), IF(2), LLF(1), HG(1), GS(2), FG(2), GQ(8), QG(8), GG(2), GL(6), RF(2), RL(2), LQ(3), AF(2), PL(1), VR(2), AG(6), GI(3), TE(1), EY(1), FR(1), TG(2), GV(1), IR(2), RR(2), IE(3), IEP(1), PQ(4), LVL(1), VLP(1), AP(2), VY(2), GR(2), GF(2), LTF(1), PG(2), FQP(1), GD(1), DG(1), DA(1), IY(2), VF(2), PR(2), QK(2), KE(1), LA(2), NK(1), KR(1), SG(2), EA(3), AA(1), GE(3), EI(1), LKP(1), KP(1), VP(2), TQ(4), EG(3), VG(2), GK(2), NG(2), NF(2), AR(3), RA(3), LN(2), LY(1), LSP(1), FWN(1), AH(1), VIY(1), GH(1), KA(1), TNP(1), IA(1), AY(1), GA(1), KL(1), KG(1), SY(1)	162	^{101}APALVYILQG RGFTGLTFPG^{120}
12S Globulin	SF(5), FP(2), LF(2), FY(1), IF(2), LY(2), NG(3), GS(1), FG(2), GQ(7), QG(7), GG(1), GL(5), KG(1), KF(2), RL(4), LQ(3), AF(2), PL(1), VR(2), AG(5), GV(3), TE(2), EY(1), TG(2), IR(2), RR(1), IE(2), IEP(1), PQ(3), LLP(1), AP(2), PG(2), PGL(1), VY(2), YL(1), GR(2), GF(2), LPG(1), AA(2), FQP(2), RA(4), HL(1), RF(1), GD(2), IA(2), GI(2), WG(1), GY(1), DG(1), DA(1), IY(1), VF(3), PR(2), QK(2), KE(3), LA(1), NK(2), SG(2), EA(2), LG(1), GE(2), EI(1), TQ(2), KP(1), PT(1), NF(2), KR(1), HG(1), LN(1), LSP(1), FWN(1), AH(1), GH(1), AR(1), ILP(1), HY(1), EG(2), TNP(1), GK(1), AY(1), EV(1), PYP(1), YP(1), KA(1)	158	^{101}APGLVYLLQG RGFTGLTLPG^{120}
Avenin-3 (Prolamin)[c]	FQP(5), PYP(1), TAP(1), VY(1), VPP(1), LNP(1), YP(1), LQQ(6), PLG(1), PL(2), IP(1), AF(1), AP(1), VP(2), RA(1), GF(1), IF(3), GI(3), GM(1), GQ(1), GG(1), QG(4), LG(1), EG(1), YVP(1), VAV(1), FAL(1), IE(1), LQ(8), LN(1), TQ(3), PP(1), PQ(5), PH(1)	65	^{131}QQFFQPQMQQ QFFQPQMQQV^{150}

[a] The number in parentheses represents the frequency of occurrence of the specific peptide sequence. [b] Fragment 101−120 was shown for 11S and 12S globulin while fragment 131−150 was shown for avenin-3. [c] The first 19 amino acids were removed from the analysis since they belonged to the immature sequence.

globulin and avenin-3 were selected for analysis using the BIOPEP database. The proteins in oats can be classified according to their solubility characteristics into four categories, namely, albumins (water-soluble), globulins (dilute aqueous salt soluble), prolamins (aqueous alcohol soluble) and glutelins (dilute acid or alkali soluble). The globulins have been shown to be the major storage proteins in oats, accounting for up to 70−80% of the total oat proteins (*22*). Although prolamins are the principal storage proteins in other cereals such as wheat, rye and barley (*22*) with the gliadin fraction being associated with celiac disease (*19*), the prolamin fraction constitutes <15% of the oat proteins, with avenin estimated at ∼2% by weight of oats (*19*). Furthermore, recent evidence suggests the safety of oats for patients with celiac disease (*31*).

Analyses of the "profiles of potential biological activity" for the three oat proteins showed that 11S globulin, 12S globulin and avenin-3 contained 162, 158, and 65 fragments nascent within their primary sequences, respectively, which matched the sequences of ACE-inhibitory or antihypertensive peptides reported in the literature (**Table 1**). Among those, GQ, QG, GL, PQ and AG were the most frequently occurring sequences common in both 11S and 12S globulin while TQ was found in higher number in 11S globulin and SF, RL and RA were observed more often in 12S globulin (**Table 1**). Aside from QG and PQ, FQP, LQQ and LQ, sequences less common in the two globulin proteins, predominated in avenin-3 (**Table 1**). Nevertheless, despite the high number of potential antihypertensive peptides, many shared overlapping peptide sequences. For example, in 11S globulin, antihypertensive peptides LQ, QG and GR were found at locations 108−109, 109−110 and 110−111, respectively, while PG, PGL and GL in 12S globulin were located in positions 102−103, 102−104 and 103−104, respectively; similarly, overlapping sequences FQP and PQ were found in avenin-3 to be at locations 134−136 and 136−137, respectively (**Table 1**). Although the large numbers of antihypertensive peptide sequences found within the oat proteins are therefore overestimates of the peptides that could actually be released from any single protein molecule, the information still provided the basis in support of further investigation of oats as a potential protein source for generation of peptides with ACE-inhibitory activity.

Proteolysis with enzymes is often used to release the bioactive peptide sequences from the intact protein molecule. Nonetheless, given the vast number of enzymes and the diversity of substrate specificity of those enzymes, it would be more efficient and effective to select a protease that would be predicted to cleave at sites yielding the antihypertensive peptides, instead of randomly choosing any enzyme for that purpose. Application of the "enzyme action" tool in the BIOPEP database on the three oat protein sequences indicated that many enzymes displayed the theoretical ability to release peptides with "antihypertensive" activity. However, only a few proteases yielded a large number of these peptides, including chymotrypsin C (33), ficin (29), pancreatic elastase (25), papain (28) and thermolysin (43) (**Table 2**). The combined action of pepsin and trypsin could theoretically release 9 of these peptides while that of pepsin, trypsin and chymotrypsin A could yield 13 active sequences, suggesting that the process of gastrointestinal digestion of oat protein might produce some antihypertensive effect (**Table 2**). Chymotrypsin C, ficin, pancreatic elastase, papain and thermolysin were predicted through the BIOPEP database to yield a much greater number of the potentially antihypertensive peptides from oat proteins. However, it should be noted that the predicted number of active peptides produced may not always correlate with the actual antihypertensive activity since the BIOPEP database classifies both *in vitro* ACE-inhibitory activity and *in vivo* antihypertensive activity as "antihypertensive" activity, and furthermore it does not consider the potency of the peptides, which will play a key role in determining the overall activity. In addition, the database does not encompass all the potential antihypertensive peptide sequences. The sequences currently in the database were acquired from the published literature, and hence the frequency of a particular enzyme being used in those studies would, to some extent, affect the number of active peptides predicted from the database.

To further narrow our choice of protease, the peptides released by chymotrypsin C, ficin, pancreatic elastase, papain and thermolysin that matched the sequences of antihypertensive peptides

Table 2. Antihypertensive Peptides Predicted Using Biopep "Enzyme Action" Tool To Be Released from Oat Proteins

protease[a]	no. of peptides			peptide sequence[c]
	11S globulin	12S globulin	avenin-3[b]	
bromelain	6	8	0	QG(5), DA(2), IY(2), LG(2), NG(1), PG(1), RA(1)
cathepsin G	2	1	0	NF(2), GF(1)
chymase	2	2	0	LLF(1), VF(2), GY(1)
chymotrypsin A	2	3	0	VY(2), VF(2), GY(1)
chymotrypsin C	15	13	5	FY(2), GL(4), VY(3), GQ(5), KE(3), KP(2), VP(2), TQ(2), KL(1), SY(1), GY(1), RL(1), HY(1), GE(2), IF(1), IE(1), GM(1)
clostripain	1	1	0	GR(1), AR(1)
ficin	14	15	0	FY(2), FG(2), QG(7), DG(2), DA(2), IY(3), EA(2), RA(4), PG(3), HG(1), IA(1)
glutamyl endopeptidase II	1	3	0	IE(2), GE(1), KE(1)
leuckocyte elastase	3	5	0	EA(2), LA(2), RA(1), GV(2), IA(1)
metridin	2	3	0	VY(2), VF(2), GY(1)
oligopeptidase B	1	1	0	QK(2)
pancreatic elastase	10	13	2	FG(2), QG(6), DA(2), EA(2), RA(3), EI(2), PG(3), KG(1), YL(1), HG(1), EG(1), PL(1)
papain	11	16	1	QG(8), DA(2), IY(2), PR(2), QK(2), LG(2), IA(2), FG(1), VR(1), PG(1), NG(1), WG(1), TE(1), SG(1), PLG(1)
plasmin	1	1	0	QK(2)
prolyl oligopeptidase	0	1	1	YP(2)
proteinase K	4	6	0	KP(2), SF(2),GI(2), KL(1), GL(1), KF(1), GY(1)
proteinase P1	10	6	0	FP(2), VR(2), GL(2), AA(2), VY(1), VG(1), GG(1), AG(1), FG(1), KE(1), RL(1), EG(1)
thermolysin	21	16	6	FY(1), LQ(6), AG(4), AP(4), VY(4), FQP(2), IY(3), LG(2), IR(2), LKP(1), VP(1), FP(2), LN(2), LSP(2), FWN(2), AR(2), FG(1), VR(1), LQQ(1)
trypsin	1	1	0	QK(2)
V-8 protease	0	1	0	GE(1)
pepsin + trypsin	4	3	2	GF(3), QK(2), NF(1), SF(1), IF(1), AF(1)
pepsin + trypsin + chymotrypsin A	6	6	1	GF(3), QK(2), NF(1), SF(1), VF(2), VY(2), GY(1), AF(1)

[a] Additional proteases that were investigated but which did not give any antihypertensive peptides include calpain, glycylendopeptidase, oligopeptidase F, pancreatic elastase II, pepsin, prolidase L. lactis s. cremoris H61 and thrombin. [b] The first 19 amino acids, which represented the signal peptide, were deleted prior to BIOPEP analysis. [c] The number in parentheses represents the sum of frequency of occurrence of the particular peptide sequence as generated by theoretical enzymatic cleavage of the three proteins investigated.

were compared. It was found that some active peptides were uniquely liberated by thermolysin; some examples include LQ, AG, AP, FQP, IR, LKP, FP, LN, LSP, FWN, AR and LQQ (**Table 2**), and among these sequences, LQ, FQP, LKP, LN, LSP and FWN have been reported to have IC_{50} below 20 μM (*11*, *24*). Hydrophobicity has been previously shown to be an important factor affecting the ACE-inhibitory potency; more specifically, higher ACE inhibition was observed when an inhibitor contained hydrophobic amino acid residues such as W, F, Y or P at the C-terminal positions, branched aliphatic side chain such as V and I at the N-terminal and/or positively charged group like R or K at the C-terminal (*2*). Besides the theoretical evidence, several studies have documented the effectiveness of thermolysin in actually producing antihypertensive peptides from different food sources such as milk proteins (*6*−*9*), insect protein (*32*), fish (*5*, *11*, *13*) and corn germ (*14*). As a result, based on the *in silico* results on oat proteins as well as previous reports of success to produce ACE-inhibitory products from various substrates, the enzyme thermolysin was chosen to hydrolyze oat protein isolate to produce ACE-inhibitory peptides with potential antihypertensive activity.

Experimental Verification of Ability of Selected Proteases To Release ACE-Inhibitory Peptides from Oat Protein Isolate. The ability of thermolysin to release ACE-inhibitory peptides from OPI was compared to the action of pepsin, subtilisin or the sequential action of pepsin and trypsin, each at an enzyme-to-substrate ratio (E/S) of 3% (w/w OPI). As shown in **Figure 1A**, subtilisin and thermolysin both resulted in DH of 1.2 mequiv/g after 18 h of hydrolysis time (t), although the latter enzyme showed a faster rate of proteolysis. On the contrary, pepsin resulted in a much lower extent of hydrolysis of 0.7 mequiv/g even after 18 h, while sequential pepsin−trypsin treatment gave a sharp increase in DH immediately after the addition of trypsin at t = 180 min and reached a DH of 1.0 mequiv/g after 18 h of hydrolysis (**Figure 1A**). Nonetheless, ACE-inhibitory activity was not always correlated with DH. Despite the lowest DH throughout 18 h of hydrolysis, hydrolysates produced with pepsin showed a gradual increase of ACE-inhibitory activity from 50% at t = 20 min to > 70% at t = 1080 min, which was similar to the ACE-inhibitory activity of hydrolysates produced by action of subtilisin or the combination of pepsin and trypsin, even though both of the latter treatments resulted in greater extent of hydrolysis than the pepsin hydrolysate (**Figure 1**). The addition of trypsin after pepsin treatment increased the DH yet the ACE inhibition of hydrolysate produced by pepsin−trypsin digestion was not higher (**Figure 1B**). Proteolysis with subtilisin and thermolysin achieved similar levels of DH after 18 h, but the ACE-inhibitory activity of hydrolysate produced with thermolysin was much higher than that hydrolyzed by subtilisin (**Figure 1B**).

The potency of thermolysin in producing ACE-inhibitory peptides was suggested by *in silico* analysis (**Table 2**) and confirmed *in vitro* (**Figure 1B**). The large number of "antihypertensive" peptides predicted to be released by thermolysin according to the BIOPEP analysis coincided with the high ACE inhibition observed *in vitro*. The high ACE-inhibitory activity was achieved within a short hydrolysis time of 20 min and did not

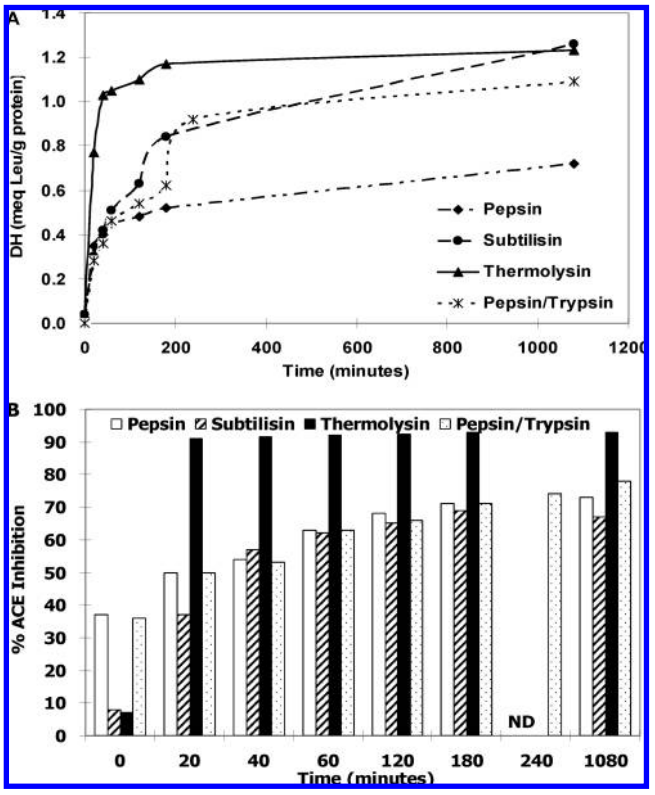

Figure 1. (**A**) Degree of hydrolysis (mequiv of Leu per g of protein) and (**B**) % ACE inhibition at 0.429 mg/mL final assay concentration of oat protein hydrolysates prepared by hydrolysis of oat protein isolate (30 mg/mL) with 3% (E/S) pepsin, subtilisin, thermolysin or pepsin/trypsin for up to 18 h at their respective optimal pH and temperature. "ND" represents data not acquired.

change upon further hydrolysis, suggesting the stability of the active peptides against further proteolytic degradation. *In silico* prediction indicated that pepsin was unsuccessful in releasing antihypertensive peptides from the major oat proteins investigated but experimental results showed some ACE inhibition (**Figure 1B**). Furthermore, the combined action of pepsin and trypsin was shown to theoretically release 9 antihypertensive peptides from 11S globulin, 12S globulin and avenin-3 (**Table 2**), comparatively more than that released by pepsin alone, yet experimental data displayed very similar ACE inhibition of the two treatments over time (**Figure 1B**). Some possible explanations for this discrepancy could be that *in silico* analysis was performed based on the reported antihypertensive peptides but many antihypertensive peptide sequences could still be unknown; in addition, the enzyme preparation used in the present study might contain other minor protease activities other than the target enzyme, which might have contributed to unpredicted release of antihypertensive peptides.

Aside from the excellent potential predicted through *in silico* analysis and the stronger ACE inhibition demonstrated in comparison with other studied enzymes, the superior potency of thermolysin to produce ACE-inhibitory peptides from various substrates other than oat proteins has been well documented in the literature. In a study on fish muscle hydrolysates, Nakajima and others (*13*) showed higher ACE-inhibitory activity of hydrolysate produced with thermolysin compared to that by pepsin−pancreatin digestion while the hydrolysis with pepsin alone showed no difference in ACE inhibition from the control without hydrolysis. Furthermore, during hydrolysis of milk casein and whey proteins, highest ACE-inhibitory activity was generally produced in the shortest hydrolysis time by thermolysin compared to proteases such as trypsin, pepsin, proteinase K or *Bacillus licheniformis*, which either required longer hydrolysis times to reach similar ACE-inhibitory activity or only achieved low ACE inhibition even after 24 h (*9*). Similarly, Hernandez-Ledesma and others (*6*) reported stronger ACE-inhibitory activity of hydrolysates of ovine and caprine β-lactoglobulin produced with thermolysin and proteinase K, especially for a hydrolysis time of less than 8 h, compared to those produced from trypsin and chymotrypsin. In addition, thermolysin produced potent ACE-inhibitory products by hydrolysis of dried bonito (*11*) as well as α-lactalbumin and β-casein (*8*). Although Vercruysse and others (*32*) have shown better ACE-inhibitory activity of insect protein hydrolysates from proteolysis with pepsin−trypsin/α-chymotrypsin compared to thermolysin, these results may have been attributed to the use of incubation temperature of 37 °C since the optimal temperature for thermolysin activity is 70 °C.

Interestingly, the OPI hydrolysates produced with thermolysin in the present study not only reached a high ACE inhibition value of 90% within 20 min of hydrolysis but also maintained this high level of ACE-inhibitory activity throughout the 18 h of proteolysis in spite of the further increase in DH (**Figure 1**). Otte and others (*9*) also showed maintenance of high ACE-inhibitory activity of milk protein hydrolysates produced with thermolysin over 24 h, while Hernandez-Ledesma and others (*6*) reported that ovine and caprine β-lactoglobulin hydrolysates produced with thermolysin showed decreased ACE-inhibitory activity with prolonged incubation time, from 81.5% at t = 0.5 h to 66.4% at t = 48 h.

The *in vitro* data thus confirmed the *in silico* results obtained from BIOPEP analysis that thermolysin was indeed a protease that could release antihypertensive peptides from oat proteins. Furthermore, OPI hydrolysates with high ACE-inhibitory activity could be produced by thermolysin within 20 min of hydrolysis, and high ACE-inhibitory activity was observed throughout prolonged proteolysis. Further research was thus conducted to investigate parameters for production of ACE-inhibitory OPI hydrolysates using thermolysin.

Effects of Thermolysin Level and Hydrolysis Time on DH and ACE-Inhibitory Activity of Oat Protein Isolate Hydrolysates. Response surface methodology using a central composite rotatable design with 13 experiments was conducted to optimize the conditions for production of OPI hydrolysates. **Table 3** shows the results for DH and ACE-inhibitory activity of hydrolysates produced using varying amounts of thermolysin and different hydrolysis times. Regression analysis of the results indicated a significant linear relationship between the amount of enzyme and DH as well as between the time of hydrolysis and DH (**Table 3**). This result is consistent with the expectation of more extensive hydrolysis in the presence of more enzyme catalyst and upon longer exposure time of the OPI substrate with the enzyme. In sharp contrast to the results for DH, however, no significant regression model could be obtained for % ACE inhibition. In other words, within the ranges of the two factors studied, namely, the amount of thermolysin (0.1−3% E/S ratio) and the hydrolysis time (10−180 min), hydrolysates were produced that exhibited similar ACE-inhibitory activity despite having differing extents of hydrolysis (**Table 3**). This indicates the potential of reducing the amount of protease and/or hydrolysis time without altering the potency of the ACE-inhibitory activity of OPI hydrolysate, consequently lowering the production cost of the process.

Therefore, the possibility of producing hydrolysates using lower amount of thermolysin (E/S of 0.3, 0.1 and 0.03%) was examined. As shown in **Figure 2A**, hydrolysate produced with 0.03, 0.1 or 0.3% thermolysin each showed a steady increase in DH as a function of time, while an increase in level of thermolysin showed a parallel increase in DH, with E/S of 0.3% displaying the

Table 3. Summary of Hydrolysis Conditions, Degree of Hydrolysis (DH), and ACE Inhibitory Activity of Oat Protein Isolate Hydrolysates (30 mg/mL) with Regression Models Generated from Response Surface Methodology

run no.	thermolysin (μg/mL)	time of hydrolysis (min)	DH (h)[a]	ACE inhibition (%)[b]
1	773	35	1.62	52
2	465	95	1.85	50
3	157	155	1.77	49
4	465	95	1.91	60
5	900	95	2.39	59
6	157	35	1.28	52
7	30	95	1.18	50
8	465	10	1.31	57
9	465	95	1.96	62
10	465	95	2.02	51
11	465	180	2.46	46
12	773	155	2.44	48
13	465	95	1.95	42

	DH (h)[a]		ACE inhibition (%)[b]	
factor	coefficient	*p*-value	coefficient	*p*-value
constant	1.938	0.000	53.000	0.000
enzyme	0.340	0.000	1.466	0.554
time	0.367	0.000	−2.820	0.271
enzyme × enzyme	−0.091	0.080	0.063	0.981
time × time	−0.041	0.389	−1.434	0.588
enzyme × time	0.083	0.203	−0.250	0.942
regression		0.000		0.815
linear		0.000		0.446
squared		0.171		0.850
interaction		0.203		0.942
lack-of-fit		0.048		0.864

[a] h = mequiv of Leu per g of protein. [b] ACE inhibition was measured at final assay concentration of 0.0714 mg/mL.

highest DH overall. When the ACE-inhibitory activity was compared, it was found that at E/S = 0.03%, there was not only no significant increase in ACE inhibition for the first 20 min but that the product promoted ACE activity, similar to that shown by unhydrolyzed OPI (at t = 0 min); however, an increase was observed at 30 and 60 min (**Figure 2B**). When the E/S was increased to 0.1%, ACE-inhibitory activity was observed immediately after 10 min and continued to increase slowly over time; on the contrary, at E/S = 0.3%, an increase in ACE-inhibitory activity was noted for the first 20 min but no further increase was observed thereafter (**Figure 2B**). These results suggest that when the E/S was too low (0.03%), the proteolytic activity was too weak to generate ACE-inhibitory peptides from OPI within a short time. On the other hand, while a high E/S (0.3%) could produce active peptides in a short time, the higher cost invested into the high level of protease did not produce much higher ACE-inhibitory activity than when E/S = 0.1%. Therefore, it appears that an E/S = 0.1% would be sufficient to produce ACE-inhibitory peptides within a reasonable time.

High Enzyme-to-Substrate Ratio Short Time (HEST) versus Low Enzyme-to-Substrate Ratio Long Time (LELT) Proteolysis of Oat Protein Isolate. Further investigation was carried out to compare the hydrolysates produced under two conditions, specifically 3% (E/S) thermolysin (the ratio most commonly reported in the literature) coupled with a short hydrolysis duration, and 0.1% (E/S) thermolysin paired with a longer incubation.

Figure 3 shows the DH and ACE-inhibitory activity for the hydrolysate produced using 3% thermolysin as a function of time. The DH increased for the first 40 min, at which point it

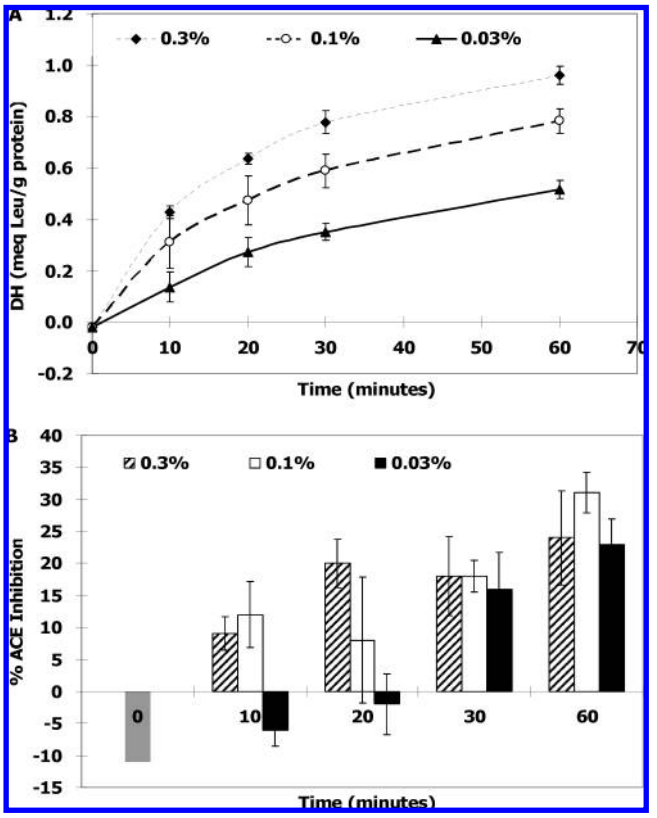

Figure 2. (**A**) Degree of hydrolysis (mequiv of Leu per g of protein) and (**B**) ACE-inhibitory activity (%) at 0.036 mg/mL final assay concentration of oat protein hydrolysates prepared by hydrolysis of oat protein isolate (30 mg/mL) with 0.3, 0.1 and 0.03% (E/S) thermolysin for up to 1 h at pH 8.0 and 70 °C. The bar in gray represents ACE-inhibitory activity of OPI without the addition of proteases at t = 0 min.

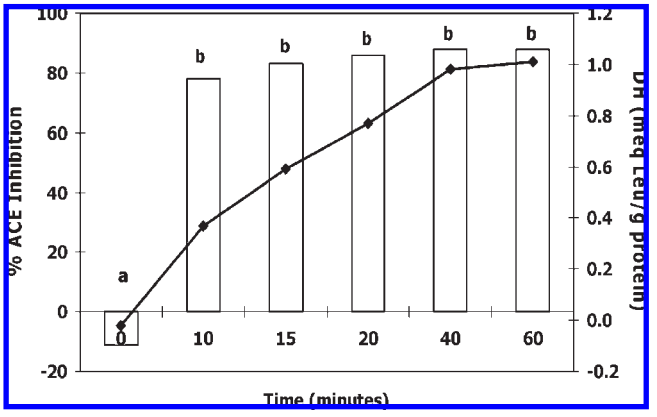

Figure 3. Degree of hydrolysis (line graph) and % ACE inhibition (bar graph) as a function of time (min) of hydrolysis of oat protein isolate (30 mg/mL) with 3% (E/S) thermolysin for up to 1 h at pH 8.0 and 70 °C. % ACE inhibition was determined at 0.429 mg/mL final assay concentration. Bars bearing different letters (a, b) are significantly different (P < 0.05).

reached a plateau value of ~1 mequiv/g, while the ACE-inhibitory activity displayed a sharp increase within the first 10 min of proteolysis, and this high activity was observed throughout the duration of hydrolysis (**Figure 3**). It should be noted that the 70% ACE inhibition value was assayed at a relatively high concentration (0.429 mg/mL) of the hydrolysate, which might have suppressed possible differences in ACE inhibition as a function of hydrolysis time. Nevertheless, the results clearly suggest that

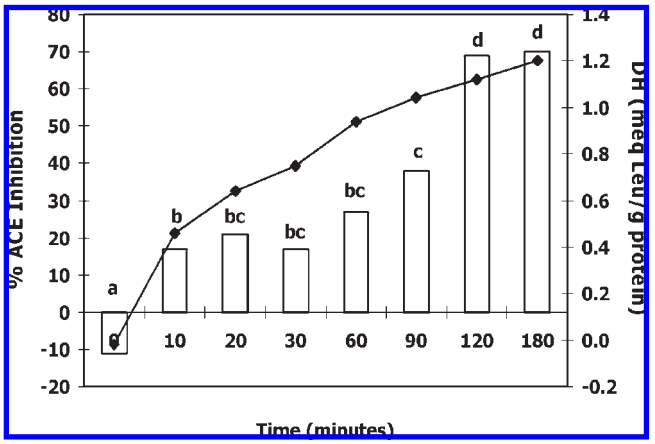

Figure 4. Degree of hydrolysis (line graph) and % ACE inhibition (bar graph) as a function of time (min) of hydrolysis of oat protein isolate (30 mg/mL) with 0.1% (E/S) thermolysin for up to 3 h at pH 8.0 and 70 °C. % ACE inhibition was determined at 0.036 mg/mL final assay concentration. Bars bearing different letters (a, b, c, d) are significantly different ($P < 0.05$).

hydrolysis conditions of E/S = 3% and hydrolysis time of 20 min (HEST) would suffice to produce peptides with high ACE-inhibitory activity from OPI.

When the hydrolysis was carried out using E/S = 0.1%, a continual increase in DH with time was observed (**Figure 4**). Interestingly, the ACE inhibition showed a slow increase for the first 90 min but a sudden increase from 35% at 90 min to 70% at 120 min was observed (**Figure 4**). The sharp increase in ACE-inhibitory activity observed from 90 to 120 min suggests that many peptides with potential activity were released. As a result, an E/S = 0.1% and a hydrolysis time of 120 min (LELT) could also produce strong ACE-inhibitory products from OPI.

In general, at any given time, OPI hydrolysates produced using thermolysin at 0.1% E/S ratio had lower DH than those produced at 3% E/S, which was expected since a larger amount of enzyme catalyst would promote more cleavage activity. However, it was surprising to note that hydrolysis at 3% E/S could yield high ACE-inhibitory activity within 10−20 min, when DH was only 0.4−0.6 mequiv/g, whereas hydrolysis at 0.1% E/S only produced high ACE-inhibitory activity when DH was >1.0 mequiv/g. These results indicate that the E/S ratio affected not only the rate of hydrolysis, but also the mechanism and cleavage sites, thereby altering the sequences and ACE-inhibitory potency of the peptides released. It is recognized that enzyme action may proceed either as a "one-by-one" reaction in which the enzyme acts on a particular substrate molecule until it is fully hydrolyzed to its end products prior to acting on a second molecule, or alternatively, the enzyme may cleave fewer sites initially on several substrate molecules to yield larger end products before further hydrolysis to smaller peptides. Although both mechanisms may arise in any hydrolysis, their occurrence is likely to be affected by the E/S ratio. Furthermore, the extent of product inhibition may differ depending on the specific peptides that are released.

The ACE-inhibitory activity assayed at 0.429 mg/mL remained at 85% for HEST hydrolysate as well as LELT hydrolysate before and after pepsin−pancreatin treatment to simulate gastrointestinal (GI) digestion (**Table 4**). The IC_{50} values before and after simulated GI digestion were 30 and 35 μg/mL for the HEST hydrolysate, and 50 and 85 μg/mL for the LELT hydrolysate (**Table 4**), suggesting slightly stronger and more stable ACE-inhibitory peptides in the hydrolysate produced using HEST compared to LELT. Although these values were still higher than the reported *in vitro* IC_{50} values of captopril (0.0072 μg/mL (*16*)),

Table 4. ACE Inhibitory Activity and IC_{50} of Oat Protein Isolate Hydrolysates Produced Using HEST (E/S = 3%, 20 min) or LELT (E/S = 0.1% and 120 min), the Hydrolysates after *in vitro* Simulated GI (Gastrointestinal) Digestion and the Hydrolysate Fractions Obtained by Ultrafiltration

	HEST		LELT	
fraction	ACE inhibition (%)[a]	IC_{50} (μg solid/mL)[b]	ACE inhibition (%)[a]	IC_{50} (μg solid/mL)[b]
hydrolysate	85	30	85	50
hydrolysate after *in vitro* GI digestion	85	35	85	85
ultrafiltered fractions				
<0.5 kDa	85	25	85	45
0.5−1 kDa	80	65	85[c]	30[c]
1−3 kDa	60	>100	−[c]	−[c]
3−10 kDa	50	>100	65	>100
>10 kDa	55	>100	55	>100

[a] ACE inhibition was measured at final assay concentration of 0.429 mg/mL for hydrolysates and 0.286 mg/mL for ultrafiltered fractions. [b] IC_{50} was determined by using cubic regression of at least 5 different concentrations ranging from 0.01−0.5 mg/mL. [c] ACE inhibition was assayed for a 0.5−3 kDa fraction.

they were substantially lower than the IC_{50} of fish muscle hydrolysate (341 μg/mL (*13*)), hake fillet hydrolysate (160 μg/mL (*12*)), soy protein digest (280 μg/mL (*16*)) and rapeseed protein hydrolysate (160 μg/mL (*15*)). Moreover, the IC_{50} values for HEST and LELT hydrolysates were comparable to IC_{50} values of milk protein hydrolysates (38 μg/mL (*6*)), dried bonito hydrolysate (29 μg/mL (*11*)), wakame hydrolysate (70 μg/mL (*18*)), cooked egg digest (9 μg/mL (*33*)), and other hydrolysates as well as purified peptides which have been demonstrated to have *in vivo* antihypertensive activity (*5*, *34*). However, *in vivo* studies would be necessary to confirm the potential antihypertensive activity of OPI hydrolysates.

The low IC_{50} of OPI hydrolysate could be related to the cleavage specificity of thermolysin, targeting at the hydrophobic amino acids such as I, L, F, Y and V (*7*) since ACE has been suggested to prefer substrate or competitive inhibitors with high hydrophobicity, especially in the C-terminal positions (*2*). Therefore, it is possible that peptides produced by hydrolyzing OPI with thermolysin contained stronger ACE-inhibitory activity than those generated using other substrates, and/or a higher number of active peptides were present in the hydrolysate, hence its potency.

Besides the promising *in vitro* evidence of the strong ACE inhibitors found in OPI hydrolysates, the ACE-inhibitory peptides produced using HEST, which employed a larger E/S ratio but shorter hydrolysis time, not only showed higher potency but also were more stable against digestion (**Table 4**). Further investigation on the peptide profiles obtained from the two processes would be necessary to explain the differences observed in ACE inhibition between HEST and LELT hydrolysates.

Further fractionation of the two hydrolysates by size revealed that small peptides were the major contributors to the ACE-inhibitory effect. IC_{50} values of 25, 65 and >100 μg/mL were observed for the <0.5, 0.5−1 and >1 kDa fractions, respectively, from HEST hydrolysate, while IC_{50} values were 45, 30 and >100 μg/mL for the <0.5, 0.5−3 and >3 kDa fractions, respectively from the LELT hydrolysate (**Table 4**). These results indicate that HEST produced more potent ACE-inhibitory peptides with sizes smaller than 0.5 kDa while LELT comprised more active peptides with sizes 0.5−3 kDa, both of which were consistent with the fact that most ACE-inhibitory peptides have been shown to contain 2−12 amino acid residues (*2*). It would be interesting to isolate and compare the peptide profiles of the active fractions from the OPH produced under HEST and LELT conditions.

Cinq-Mars and Li-Chan (*12*) have shown an improvement in ACE-inhibitory activity by ultrafiltration of hake hydrolysate, from an IC_{50} value of 160 μg/mL for the unfractionated hydrolysate, to 40 μg/mL for the <10 kDa ultrafiltered fraction. Nevertheless, in the present study, the unfractionated hydrolysate was shown to exhibit equivalent or stronger ACE inhibition than the fractionated counterparts. This finding could be advantageous in large scale production where a simpler process would be preferred since the cost would be minimized.

In conclusion, the potential of producing potent ACE inhibitors from hydrolysis of oat protein isolate was assessed *in silico* and confirmed *in vitro* with thermolysin. Different hydrolysis conditions were shown experimentally to successfully produce ACE-inhibitory products from oat protein isolate, with the hydrolysate produced by thermolysin under high enzyme-to-substrate ratio (3%) short time (20 min) conditions showing slightly stronger activity than hydrolysate produced using a low enzyme-to-substrate ratio (0.1%) long time (120 min) process. The ACE-inhibitory activity was stable to prolonged hydrolysis time as well as simulated gastrointestinal digestion. Further fractionation of the hydrolysates by size did not improve ACE inhibition implying a simple process of hydrolysis would be sufficient to produce functional products. The promising results from the present study warrant further research to develop potent ACE inhibitors from oat protein hydrolysates, to identify the peptide sequences in hydrolysates produced under different conditions, and to assess their *in vivo* antihypertensive efficacy.

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