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ACE-I inhibitory properties of hydrolysates from germinated and ungerminated *Phaseolus lunatus* proteins

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Abstract

Phaseolus lunatus protein concentrates and the proteases Alcalase(R) and Pepsin-Pancreatin were used for the production of protein hydrolysates that inhibit angiotensin-I converting enzyme (ACE). Protein concentrate obtained from germinated and ungerminated seeds flour was hydrolyzed with Alcalase(R) at enzyme/substrate ratio (E/S) 1/10 and during 0.5 and 2.0 h, respectively. On the other hand, protein concentrate obtained from ungerminated (E/S: 1/10) and germinated (E/S: 1/50) seeds flour was sequentially hydrolyzed with Pepsin-Pancreatin during 1.0 and 3.0 h, respectively. Peptide fractions with ACE inhibitory activity in a range of 0.9 to 3.8 μg/mL were obtained by G-50 gel filtration chromatography and high- performance liquid chromatography C18 reverse phase chromatography. The observed amino acid composition suggests a substantial contribution of hydrophobic residues to the peptides' inhibitory potency, which potentially acts via blocking of angiotensin II production. These results show that *P. lunatus* seed proteins are a potential source of ACE inhibitory peptides when hydrolyzed with Alcalase(R) and Pepsin-Pancreatin.

Keywords: *P. lunatus*; hydrolysates; peptides; ACE inhibitory activity.

Practical Application: Legume proteins can enzymatically produce bioactive specific peptides for use in functional foods.

1 Introduction

Hypertension is a worldwide problem that affects 15-20% of all adults. Its treatment is one of the major risk factors for the development of cardiovascular disease, stroke, and the end stage of renal disease (Kim & Byun, 2012). The angiotensin-I converting enzyme (ACE) in the renin-angiotensin system plays an important physiological role in regulating blood pressure in the human body. It catalyzes the cleavage of the C-terminal dipeptide from inactive angiotensin I to become active angiotensin II and inhibits the activity of the vasodilator bradykinin. Consequently, this concerted action endows ACE with a crucial role in controlling blood pressure. Therefore, it is capable of suppressing the elevation of blood pressure by inhibiting the catalytic action of ACE (Cao et al., 2010). Many synthetic ACE inhibitors including Captopril, Enalapril, and Lisinopril among others present some undesirable side effects such as cough, loss of taste, renal impairment and angioneurotic oedema (Kim & Byun, 2012).

Proteins are fundamental and integral food compounds providing nutritional and functional properties, also many food's proteins possess specific biological activities (Wanasundara et al., 2002). Bioactive peptides can range from 2 to 20 amino acid residues that have a positive impact on body functions which is based on their amino acid composition and sequence (Liu et al., 2012). Bioactive peptides can be absorbed in the intestine and enter the blood stream directly, which ensures their bioavailability *in vivo* and physiological effect at the target site. Research has been carried out due to their potential nutraceuticals in relation to the development of functional foods. Such regulatory peptides can be released by enzymatic hydrolysis of proteins from foods (Wanasundara et al., 2002) *in vivo* or *in vitro,*this because peptides are not active within the origin protein. Bioactive peptides can act as immunoregulatory, ACE inhibitory, opioid, antimicrobial, and antioxidant. Among these, ACE inhibitory peptides have received more attraction due to having significant effect on prevention and treatment of hypertension (Memarpoor-Yazdi et al., 2012).

Daily use of food that contains peptides with potent ACE inhibitory activity instead of synthetics could be effective in maintaining blood pressure at a healthy level without exhibit side effects (Cao et al., 2010). The use of enzyme technologies for protein recovery and modification has led to the production of a broad spectrum of food ingredients and industrial products. Proteases from different sources are commonly employed to obtain a more selective hydrolysis due to their specificity for peptide bonds adjacent to certain amino acid residues. Enzymatic hydrolysis of food proteins is an efficient way to recover potent bioactive peptides (Nalinanon et al., 2011). In recent years, ACE-inhibiting peptides from food sources are promising natural bio-functional alternatives to the synthetic drugs. Many ACE-inhibiting peptides have been discovered in enzymatic hydrolysates of different food-source proteins, and they were to be applied in the prevention of hypertension and in the initial treatment of mildly hypertensive individuals.

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Therefore, searching for natural ACE inhibitors as alternatives to synthetic ones is of greater interest for safe and economical use as pharmaceuticals (Liu et al., 2012).

Lima bean (*Phaseolus lunatus*) is grown in tropical regions such as Mexico. Its seeds have high protein (210-260 g/kg) and high carbohydrate contents (550-640 g/kg), low fat (10 - 23 g/kg) and fiber levels (32-68 g/kg), high levels of minerals such as K, Zn, Ca and Fe, and low levels of Na and P (Oshodi & Aletor, 1993). Use of plant products as functional ingredients in foods is rising rapidly, due both to their cost-effectiveness and the improvements they provide in product nutritional quality. *Phaseolus lunatus* is a promising source of protein hydrolysates with ACE inhibitory properties due to aminoacidic composition. For the above mentioned, the present study objective was to purify ACE inhibitory peptides from *P. lunatus* protein hydrolysates and quantify the resulting peptides' amino acid composition.

2 Materials and methods

2.1 Seeds and chemicals

Phaseolus lunatus L. seeds were purchased at a local market in Mérida, Yucatán, Mexico. Any impurities were manually removed from the seeds. For seeds germination, seeds were washed with water, soaked in distilled water and immersed for 1 min in 20 mL sodium hypochlorite solution (0.2 w/v), and washed again three times in 25 mL distilled water. They were then germinated for 48 h in a germination room in darkness at 16-18°C, with moisture levels maintained by spraying with distilled water every 8 h. Flours from ungerminated and germinated seeds were prepared by cleaning the seeds, milling them in a Mykros impact mill, and screening through a 20 mesh screen (0.85 mm pore opening). ACE, Hippuryl-L-histidyl-L-leucine, 2,4,6-trichloro-s-triazine, o-phthaldialdehyde, serine standard, gastric porcine pepsin, and pancreatin were purchased from Sigma. Alcalase$^{(R)}$ 2.4 L was obtained from Novo Nordisk, Bagsvaerd, Denmark. All other chemicals were analytical grade.

2.2 Protein concentrates from ungerminated and germinated seeds

Protein concentrates (PC) were extracted from the ungerminated and germinated seeds according to Chel-Guerrero et al. (2002). Three, 1 Kg batches of flour were processed by preparing flour: water (1:6 w/v) suspension, and adjusting pH to 11 with 1N NaOH. After soaking for 1 h, the suspensions were milled in a disk mill and passed through 80 and 100 mesh screens to separate the fiber-containing solid fraction from the liquid fraction, which contains the protein and starch. Residual solids were washed 5 times with distilled water at a 1:3 v/v ra- tio and then passed through 150 mesh screen to eliminate the finest fiber. Wash water was mixed with the super- natants from the initial suspension and this solution al- lowed to sediment for 30 min to recover the starch fraction and separate the solubilized protein. The pH of the solubilized proteins was adjusted to the isoelectric point (4.5) with 1N HCl. The suspension was then centrifuged at 1317 × g for 12 min (Mistral 3000i, Curtin Matheson Sci.), the supernatants discarded and the precipitates freeze-dried at –47°C and 13×10^{-3} mbar.

2.3 Enzymatic hydrolysis

Hydrolysis conditions for obtaining hydrolysates analyzed in this study (H1, H2, H3 and H4) were selected from sixteen treatment of a previous study (Chel-Guerrero et al., 2012). In this study the protein hydrolysate production with ACE-I inhibitory activity was analyzed with a 2^3 factorial design and evaluated factors were protein concentrate source (ungerminated or germinated seeds of lima bean), E/S ratio (1/50 or 1/10) and hydrolysis time (0.5 or 2 h for Alcalase$^{(R)}$; 1 or 3 h for Pepsin-Pancreatin).

Alcalase protein hydrolysis

Following the method of Li et al. (2005), protein concentrate suspensions (4 g protein in 100 ml water) were prepared from the ungerminated and germinated seed flours. Hydrolysis of these suspensions was done using Alcalase$^{(R)}$ 2.4 L, adjusted to 0.3 AU/g by dilution with distilled water. Protein concentrate obtained from germinated and ungerminated seeds flours was hydrolyzed at enzyme/substrate ratio (E/S) (1/10) and during 0.5 and 2.0 h, respectively. Hydrolysis was done in a pH stat, 50 mL vessel, by adding 0.3 AU/g Alcalase$^{(R)}$. The reaction was run at 50°C, pH adjusted to 7.0 with 0.1 N NaOH and the suspension kept under constant agitation. Hydrolysis was finalized by inactivating the protease through heating to 85°C for 10 min. The hydrolysates were centrifuged at 13211 x *g* for 20 min at 4°C (Beckman) and the supernatants freeze-dried (Labconco) for later analysis.

Pepsin-pancreatin protein hydrolysis

Protein hydrolysis was done with a sequential pepsin-pancreatin system involving digestion with pepsin (P7000, Sigma) for half the hydrolysis time followed by incubation with pancreatin (P1500, Sigma) for the other half (Megías et al., 2004). Protein concentrate obtained from ungerminated (E/S: 1/10) and germinated (E/S: 1/50) seeds flours was hydrolyzed during 1.0 and 3.0 h, respectively. Hydrolysis was done in a 50 mL reaction vessel equipped with a stirrer, thermometer and pH electrode. Hydrolysis was run at 37°C using a 4% (w/v) concentration for both enzyme solutions, and pH adjusted to 2 with 0.1 N HCl for pepsin and to 7.5 with 0.1 N NaOH for pancreatin. The hydrolysis reaction was stopped by heating to 80°C for 20 min. The hydrolysates were centrifuged at 13211 × *g* for 20 min at 4°C (Beckman) and the supernatants freeze- dried (Labconco) for later analysis.

2.4 Degree of hydrolysis

DH was calculated by calculating free amino groups with o-phthaldialdehyde (Nielsen et al. 2001): $DH = h/h_{tot} * 100$, where h_{tot} is the total number of peptide bonds per protein equivalent, and h is the number of hydrolyzed bonds. The h_{tot} factor is dependent on raw material amino acid composition.

2.5 ACE inhibitory activity

ACE was prepared according to Hayakari et al. (1978) Rabbit lungs were used as starting material and the aliquots obtained were stored at –20°C for no more of two months. ACE inhibition

from GS and IS aliquots, was evaluated in 20 µL of solution from these samples. Determination of IC_{50} was performed by plotting log concentration of GS and IS protein (mg/mL) vs. percentage of ACE-I activity.

2.6 G-50 gel filtration chromatography

1 mL of the protein hydrolysates was injected into a Sephadex G-50 gel filtration column (2 cm × 55 cm) at a flow rate of 10 mL/h of 50 mM ammonium bicarbonate (pH 9.1). The resulting fractions were collected for assaying ACE inhibitory activity according to Hayakari et al. (1978). Peptide molecular masses were determined by reference to a calibration curve created by running molecular mass markers on the Sephadex G-50 under conditions identical to those used for the test samples. Molecular mass standards were cytochrome C (12 384 Da), bacitracin A (1 422.69 Da), Val-angiotensin (1031 Da), RKEVY (693.8 Da) and TQ (247.8 Da). Protein elution was monitored at 280 nm.

2.7 HPLC C18 chromatography

The fractions isolated with the Sephadex G-50 column were redissolved in deionized water and injected into a preparative HPLC (Agilent, Model 1110) reverse-phase column (C18 Hi-Pore RP-318, 250 mm ×10 mm BIO-RAD column). The injection volume was 100 µL, and the sample concentration was 20 mg/mL. Elution was achieved by a linear gradient of acetonitrile in water (0-30% in 50 min) containing 0.1% trifluoroacetic acid at a flow rate of 4 mL/min and 30 °C [10]. Elution was monitored at 215 and 280 nm, and the resulting fractions were collected for assay of ACE inhibitory activity as described previously (Hayakari et al., 1978). The fractions with higher ACE inhibitory activity were injected in an analytical HPLC reverse phase column (C18 Hi-Pore RP-318, 250 mm × 4.6 mm BIO-RAD column) for the analysis of ACE inhibitory activity in individual peaks. The injection volume was 50 µL, and the sample concentration was 2 mg/mL. Elution was achieved by a linear gradient of acetonitrile in water containing 0.1% trifluoroacetic acid at a flow rate of 1 mL/min at 30 °C and was monitored at 215 and 280 nm.

2.8 Amino acid composition

Protein amino acid composition was determined for the hydrolysates and the peptides purified by gel filtration chromatography and HPLC according Alaiz et al. (1992),were separated using HPLC with a reversed-phase column (300 × 3.9 mm, Nova Pack C_{18}, 4µm; Waters). Tryptophan was determined according Yust et al. (2004).

2.9 Statistical analysis

All results were analyzed in triplicate using descriptive statistics with a central tendency and dispersion measures. One-way ANOVAs were performed to evaluate *in vitro* ACE inhibitory activity. A Duncan's multiple range test was used to determine differences between treatments. All analyses were performed according to Montgomery (2001) and processed using the Statgraphics Plus version 5.1 software.

3. Results and discussion

3.1 ACE inhibitory activity of protein hydrolysates from P. lunatus

Enzymatic hydrolysis is widely used to produce food-grade protein hydrolysates and to release bioactive peptides from their protein precursors. In the present study, hydrolysis of germinate seeds of *P. lunatus* concentrate with Alcalase(R) resulted in a maximum DH of 30.34% with a hydrolysis time of 0.5 h and E/S of 1/10 (H1). However, a higher DH (51.28%) was obtained to hydrolyze protein concentrate from ungerminated seeds for 2h at the same hydrolysis conditions (H2). Hydrolysis with the endoprotease Alcalase(R) increased DH at longer hydrolysis time; registering the higher DH in the protein concentrate from ungerminated seeds. It is possible that denaturation during the concentration process produced a better molecular orientation in protein concentrate from ungerminated seeds than in germinated seeds, which would have enhanced the enzyme-substrate interaction. ACE inhibitory activity of *P. lunatus* protein hydrolysates obtained with Alcalase(R) registered IC_{50} values of 610 and 560 µg/mL for H1 and H2, respectively. Hydrolysis with the endoprotease Alcalase(R) increased ACE inhibitory activity at higher DH, registering the higher biological potential in the protein concentrate from ungerminated seeds.

Hydrolysis of protein concentrate from ungerminated seeds with pepsin-pancreatin with E/S: 1/10 and hydrolysis time of 1 h generated a DH of 32.16% (H3). However hydrolysate with E/S of 1/50 and 3 h of reaction from protein concentrate of germinated seeds had a DH of 29.28% (H4). Hydrolysis with the endoproteases pepsin-pancreatin reduced DH at longer hydrolysis time; registering the higher DH in the protein concentrate from ungerminated seeds with enzyme/substrate ratio of 1/10. ACE inhibitory activity of pepsin-pancreatin hydrolysates, measured and calculated as IC_{50}, was 250 and 280 µg/mL for H3 and H4, respectively, registering the higher biological potential in the protein concentrate from ungerminated seeds (Table 1).

All the evaluated *P. lunatus* protein hydrolysates, those from Alcalase(R) or pepsin-pancreatin hydrolysis, exhibited ACE

Table 1. Degree of hydrolysis and ACE inhibitory activity of protein hydrolysates from *P. lunatus*.

Treatment	Enzyme	Substrate	Enzyme/ substrate ratio	Hydrolysis time (h)	DH (%)	IC_{50} (µg/mL)
H1	Alcalase (R)	Germinated seeds	1/10	0.5	30.34	610
H2		Ungerminated seeds	1/10	2	51.28	560
H3	Pepsin-Pancreatin	Ungerminated seeds	1/10	1	32.16	250
H4		Germinated seeds	1/50	3	29.28	280

inhibitory activity. The best IC_{50} value was produced using protein concentrate from ungerminated seeds sequentially hydrolyzed with pepsin-pancreatin. The IC_{50} value ranges observed in the present study encompass protein hydrolysate IC_{50} values from vegetable protein sources reported in the literature: 180 μg/mL for maize gluten (Kim et al., 2004); same value for chickpea legumin (Yust et al., 2003); 1360 μg/mL for chickpea total protein (Vermeirssen et al., 2002); 300-1200 μg/mL for buck- wheat protein (Li et al., 2002) and 640 g/mL for mungo bean protein (Li et al., 2005). The IC_{50} values reported for hydrolysates from animal protein sources differ noticeably: 8-11.2 μg/mL for milk protein (Gobbetti et al., 2000) and 20-74.4 μg/mL for egg white protein (Miguel et al., 2007).

3.2 G-50 gel filtration chromatography

Gel filtration chromatography (Sephadex G-50 column) was used to generate a molecular weight profile of the *P. lunatus* protein hydrolysates. The profile was typical of a protein hydrolysate formed by a pool of peptides, with gradually decreasing molecular masses. Elution volumes between 57 and 104 mL included free amino acids and peptides with molecular masses ranging from 0.25 to 1.42 kDa. This range was fractionated into six fractions (1 to 6) and ACE inhibitory activity determined for each at 5μg of protein concentration. Fractions with elution volumes smaller than 57 mL and greater than 104 mL were not analyzed because they largely included peptides with high molecular weights, as well as free amino acids.

Figure 1 showed the elution profiles of Alcalase(R) protein hydrolysates (H1 and H2). The elution profile of H1 from germinated seeds, (Figure 1a) showed five fractions with ACE inhibitory activity. ACE inhibitory activity (%) in the five fractions ranged from 8.65 to 21.08% and differed ($p<0.05$) between fractions. The highest ACE inhibitory activity was observed in fractions F1-4 (21.08%) and F1-5 (17.84%), which were not statistically different ($p<0.05$). Their molecular masses were approximately 0.70 kDa and 0.25 kDa, respectively. Figure 1b showed the elution profile of H2 from ungerminated seeds. ACE inhibitory activity was registered in only two fractions: F2-3 (14.6%) and F2-4 (24.32%) with molecular masses of 0.70 and 0.25 kDa respectively.

Figures 1c and 1d showed the elution profiles of pepsin-pancreatin protein hydrolysates (H3 and H4). The elution profile of H3 (from ungerminated seeds) showed three fractions with ACE inhibitory activity. Biological activity in the three fractions ranged from 13 to 34% and differed ($p<0.05$) between fractions. The highest ACE inhibitory activity was observed in fraction F3-4 (34%) (Figure 1c). Figure 1d showed the elution profile of H4 (from germinated seeds). ACE inhibitory activity (%) was registered in fractions F4-1 (29.73%), F4-2 (45.95%), F4-5 (40.54%) and F4-6 (9.73%). F4-1 and F4-2 registered molecular masses between 0.7 and 1.03 kDa while that F4-5 and F4-6 showed molecular masses of 0.25 and 0.70 kDa.

3.3 HPLC C18 chromatography

Fractions F1-4, F1-5 as well as F2-3 and F2-4 of the Alcalase(R) protein hydrolysates and F3-2, F3-4 as well as F4-2 and F4-5 of the Pepsin-Pancreatin protein hydrolysates purified in a Sephadex G- 50 gel filtration column were pooled and analyzed using preparative RP-HPLC-C18 to produce a chromatographic profile from mass-transfer between stationary and mobile phases. The peptides were relatively pure, although a small shoulder still appeared behind the peaks in the chromatogram (data not showed). To be F2-4 and F3-4 that provided the best resolution in the elution profile and acceptable ACE inhibition of ACE (24.32% and 34%, respectively) it were selected to further analysis. For F2-4 from Alcalase(R) hydrolysis, eluted peptides were divided in three fractions, F2-4A (10-15 min elution time), F2-4B (15-20 min elution time) and F2-4C (20-25 min elution time). Enough material from each fraction was collected in successive analyses to determine ACE inhibitory activity, resulting with ECA inhibition of 25.84% (F2-4C), 14.27% (F2-4A) and F2-4B had the highest ACE inhibitory activity (30.67%). For F3-4 from Pepsin-Pancreatin hydrolysis, eluted peptides were divided in three fractions too: F3-4A (10-15 min elution time), F3-4B (15-20 min elution time) and F3-4C (20-25 min elution time). Again, the peptides that eluted last from the reverse phase column had the highest ACE inhibitory activity: F3-4C (63.12%) had the highest ACE inhibitory activity and F3-4B and F3-4A the lowest ACE inhibition with 43.87 and 29.03% respectively. As previously observed with chickpea and sunflower protein hydrolysates; the peptides that eluted last from the reverse phase column had the highest ACE inhibitory activity. It has been suggested that ACE inhibitory peptides are rich in hydrophobic amino acids, which results in a higher retention in hydrophobic chromatography column (Pedroche et al., 2002; Megías et al., 2004).

Further purify ACE inhibitory peptides, F2-4B and F3-4C that provided the highest inhibition of ACE, were applied to an analytical C18 reverse phase column. For F2-4B fraction obtained from Alcalase® hydrolysis, eluted peptides were divided in eight fractions, (F2-4B1 to F2-4B8). Enough material from each fraction was collected in successive analyses to determine ACE inhibitory activity. Overall, F2-4B6 (40.6%), F2-4B7 (48.8%) and F2-4B8 (32.4%) had the highest ACE inhibitory activity with IC_{50} values of 2.9, 1.7 and 3.8 μg/mL, respectively and F2-4B1 to F2-4B5 had an ACE inhibition value from 20 to 40%.

For F3-4C fraction from Pepsin-Pancretin hydrolysis, eluted peptides were separated in six fractions, (F3-4C1 – F3-4C6). Overall, F3-4C3 (37.4%), F3-4C4 (43.2%) and F3-4C5 (58.7%) had the highest ACE inhibitory activity with IC_{50} values of 3.4, 2.7 and 0.9 μg/mL, respectively and F3-4C1, F3-4C2 and F3-4C6 had the lowest value (8.2, 14.8 and 29.1% respectively).

For both F2-4B and F3-4C fractions, the best ACE inhibition from fracctions purified by a second step of HPLC reverse fase chromatography were obtained from those collected intermediately. Similar behavior was observed in the purification of peptides from protein hydrolysates of chickpea seeds using the Alcalase(R) enzyme (Pedroche et al., 2002; Yust et al., 2003) or of sunflower seeds using Pepsin-Pancreatin enzymes (Megías et al., 2004).

3.4 Amino acid composition

Peptide fractions with the highest ACE inhibitory activity were analyzed to produce an amino acid profile. During hydrolysis, asparagine and glutamine partially converted to aspartic acid and

glutamic acid, respectively; the data for asparagine and/or aspartic acid were therefore reported as Asx while those for glutamine and/or glutamic acid were reported as Glx. The higher ACE inhibitory activity exhibited by the *P. lunatus* protein hydrolysates from ungerminated seeds H2 (560µg/mL) and H3 (250µg/mL) was probably due to its higher concentration of hydrophobics amino acids as Pro (1.33 and 2.25 mg/100g of protein) (Table 2). The same behavior was observed to compare the amino acids content of gel filtration chromatography fractions from *P. lunatus* hydrolysates. Peptide fractions as F2-4 and F3-4, which were

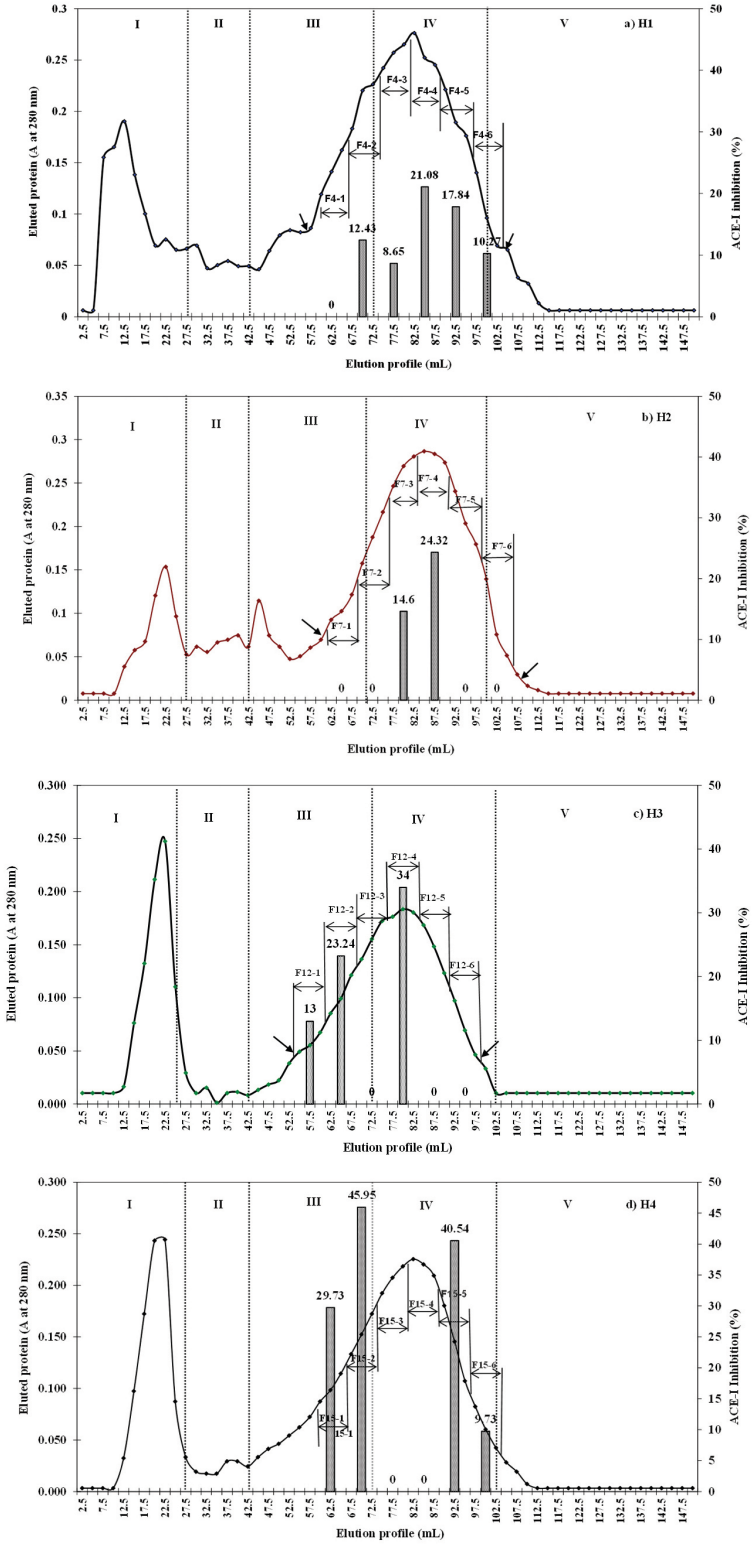

Figure 1. Elution profile of the Alcalase(R) (H1 from germinated seeds and H2 from ungerminated seeds) and pepsin-pacreatin (H3 from ungerminated seeds and H4 from germinated seeds) protein hydrolysates purified in a Sephadex G-50 gel filtration column.

Table 2. Aminoacids content of protein hydrolysates and isolated fractions from *P. lunatus* seeds.

Amino Acid	Content (g/100g)																	
	H1	H2	H3	H4	F1-4	F1-5	F2-3	F2-4	F3-2	F3-4	F4-2	F4-5	F2-4B6	F2-4B7	F2-4B8	F3-4C3	F3-4C4	F3-4C5
Asx	9.24	8.63	12.82	7.32	11.66	15.18	12.5	9.87	15.72	12.96	15.7	13.42	11.4	10.25	13.44	17.49	15.66	12.53
Glx	13.08	14.46	15.41	14.37	10.64	13.25	14.73	12.24	17.5	13.49	18.35	15.02	8.67	8.57	10.63	9.56	7.21	5.98
Ser	8.15	7.68	7.5	8.12	7.1	2.34	7.69	6.4	6.75	6.15	7.32	2.85	9.83	7.99	8.36	5.75	6.93	4.94
His	3.95	3.93	3.13	3.81	3.22	ND	2.73	4.22	2.46	1.63	2.27	1.24	4.04	2.29	2.11	2.04	2.04	1.73
Gly	4.87	4.98	4.9	5.13	4.99	4.16	5.5	4.2	5.85	4.76	5.74	3.68	6.11	5.65	4.8	4.14	3.51	3.87
Thr	5.02	4.97	4.59	5.14	3.68	3.32	4.91	4.67	5.26	3.85	4.95	2.98	5.87	4.89	4.67	4.03	2.53	2.63
Arg	7.06	5.91	6.1	6.44	8.35	7.66	5.57	5.61	3.77	6.06	4.88	4.93	2.19	1.83	2.19	1.66	3.14	2.14
Ala	5.85	6.14	5.26	5.94	5.82	5.21	6.41	12.56	9.28	6.23	6.93	6.97	8.72	8.46	9.62	4.58	4.19	3.56
Pro	0.87	1.33	2.25	1	3.01	ND	2.77	2.7	5.39	3.5	3.86	ND	1.43	3.29	2.13	2.1	10.95	14.45
Tyr	4.76	4.11	4.04	4.63	6.67	ND	2.78	4.03	0.05	4.33	0.34	ND	2.75	2.2	1.35	1.35	0.81	ND
Val	5.45	5.8	4.93	5.75	4.14	2.73	5.1	5.2	5.1	4.94	5.2	4.65	5.41	6.82	5.85	14.92	4.85	3.14
Met	1.93	1.78	1.05	1.64	0.8	ND	0.22	0.65	0.43	0.36	0.26	ND	0.43	0.35	0.23	0.58	3.75	10.82
Cys	0.82	0.75	0.72	0.89	0.62	0.89	0.24	0.5	0.83	0.18	0.89	ND	0.17	0.44	1.23	ND	ND	ND
Ile	4.52	4.67	4.11	4.75	3.33	2.56	4.23	7.7	5.63	4.84	4.86	4.89	10.07	10.87	9.88	5.81	6.34	7.94
Leu	9.58	9.76	8.82	9.91	9.29	13.1	10.22	7.03	6.61	11.8	8.22	14.81	11.81	10.59	12.09	13	11.04	10.81
Phe	6.49	6.45	6.02	6.74	9.43	12.05	4.88	6.87	3.03	8.21	3.54	13.28	5.18	7.61	4.89	5.93	7.08	3.74
Lys	7.72	8.03	7.62	7.8	5.15	0.85	8.26	4.42	4.56	4.96	6.66	4.94	3.7	3.56	3.28	4.89	4.53	4.53
Trp	0.63	0.65	0.74	0.64	2.12	16.71	1.27	1.15	1.79	1.79	0.02	6.35	2.23	4.25	3.32	2.25	5.53	7.23
Amino acid distribution																		
Hydrophobic	35.33	36.6	33.17	36.36	37.94	52.36	35.09	43.85	37.26	41.66	32.89	50.96	45.29	52.22	47.96	49.16	53.74	61.69
Neutral	23.62	22.5	21.75	23.91	23.05	10.71	20.86	19.79	18.74	19.25	19.25	9.51	24.72	21.16	20.4	15.27	13.78	11.43
Hydrophilic	45.61	40.96	45.08	39.74	39	36.94	45.04	36.36	43.99	39.09	47.87	39.54	30	26.5	31.64	35.64	32.57	26.91

selected to further purification, registered a hydrophobic amino acid content of 43.85 and 41.66%, respectively. Amino acids as Ala, Val, Ile and Leu were founded in these fractions in higher proportions (Table 2). Amino acids content of fractions with higher ACE inhibitory activity (F2-4B7, F3-4C5) purified by analytical RP-HPLC C-18 chromatography from fractions F2-4B and F3-4C obtained by preparative RP-HPLC C-18 chromatography registered the higher hydrophobic amino acids content (52.22 and 61.69%, respectively) of all purified fractions. F2-4B7 registered the higher content of amino acids such as Pro (3.29 g/100g), Ile (10.87 g/100g) and Phe (7.61 g/100g) of purified fractions from F2-4B, while that F3-4C5 registered the highest levels of hydrophobic residues such as Pro (14.45%), Met (10.82%) and Trp (7.23%) (Table 2).

According to Byun & Kim (2002). ACE inhibitory peptides that are derived from proteins are regarded as competitive substrates for ACE. The inhibitory activity is mainly dependent on a specific peptide structure. Structure-activity relationships among different peptide inhibitors of ACE indicate that binding to ACE is strongly influenced by the **C** -terminal tripeptide sequence of the substrate. Ondetti & Cushman (1982) proposed a binding model for interactions between the substrate and active site of ACE. The C -terminal tripeptide residues may interact with the subsites S1, S1', and S2' at the active site of ACE. ACE appears to prefer substrates or competitive inhibitors that contain hydrophobic amino acid residues at the three positions of the C -terminal (Byun & Kim, 2002). Matsumura et al. (1993) isolated four ACE inhibitory peptides from an autolysate of bonito bowels. The IC_{50} values of Leu-Arg-Pro, Ile-Arg-Pro, Val-Arg-Pro, and Ile-Lys-Pro were 1.0, 1.8, 2.2, and 2.5 μM, respectively. These results suggest that the proline residue should be placed at the C-terminal for exhibiting the ACE inhibitory activity. Fujita & Yoshikawa (1999) reported that Leu-Lys-Pro-Asn-Met that is derived from fish protein might be hydrolyzed further to produce Leu- Lys-Pro, which has an 8-fold higher ACE inhibitory activity relative to the parent peptide. The above-mentioned show the importance of hydrophobic residues in the biological potential of the peptide fractions from *P. lunatus*.

Moskowitz (2002) proposed a model explaining the clinical superiority of hydrophobic ACE inhibitory drugs relative to hydrophilic ones: all ACE inhibitors bind to the C-terminal catalytic site, but only hydrophobic ones bind to the occluded N-terminal catalytic site and are therefore better at blocking angiotensin II production. This would also explain why hydrophobic ACE inhibitors have specific local benefits such as organ damage prevention, in addition to reducing blood pressure. The high hydrophobic amino acid content in peptides fractions obtained of protein hydrolysates produced with concentrates from ungerminated seeds may therefore make a substantial contribution to these fractions' ACE inhibitory activity by blocking angiotensin II production.

4 Conclusion

Extensive hydrolysates with ACE inhibitory activity were generated using Alcalase(R) and Pepsin-Pancreatin proteases using *P. lunatus* as substrate. Peptide fractions were purified using gel filtration chromatography followed by HPLC chromatography. The ACE inhibition values (IC_{50}= 0.9-3.8μg/mL) of *P. lunatus* peptide fractions were similar to those reported for many other natural ACE inhibitory peptides. The results suggested a substantial contribution of hydrophobic residues to the peptides' inhibitory potency. Then these peptides can be used for elaboration of functional foods, thus widening the possible uses of *P. lunatus* proteins seeds.

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