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Lima Bean (*Phaseolus lunatus*) Protein Hydrolysates with ACE-I Inhibitory Activity

Luis Chel-Guerrero[1], Mario Domínguez-Magaña[2], Alma Martínez-Ayala[3], Gloria Dávila-Ortiz[2], David Betancur-Ancona[1]

[1]Facultad de Ingeniería Química, Universidad Autónoma de Yucatán, Mérida, México; [2]Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Miguel Hidalgo, México; [3]Centro de Investigación en Biotecnología Aplicada, Instituto Politécnico Nacional, Tlaxcala, México.
Email: bancona@uady.mx

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ABSTRACT

Several protein sources can be used to produce bioactive peptides with angiotensin I-converting enzyme (ACE) inhibitory activity. Protein concentrates from ungerminated and germinated lima bean *Phaseolus lunatus* seed flours were hydrolyzed with Alcalase 2.4 L or pepsin-pancreatin sequential hydrolysis, and ACE inhibitory activity measured in the different hydrolysis treatments. Protein hydrolysate production was analyzed with a 2^3 factorial design with four replicates of the central treatment. Evaluated factors were protein concentrate source (ungerminated seeds, PC_1; germinated seeds, PC_2), enzyme/substrate ratio E/S (1/50 or 1/10) and hydrolysis time (0.5 or 2.0 h for Alcalase; 1 or 3 h for pepsin-pancreatin). Degree of hydrolysis (DH) was high for the Alcalase hydrolysates (24.12% - 58.94%), but the pepsin-pancreatin hydrolysates exhibited the highest ACE inhibitory activity (IC_{50} = 0.250 - 0.692 mg/mL). Under the tested conditions, the hydrolysates with the highest ACE inhibitory activity were produced with sequential pepsin-pancreatin using either PC_1 at 1 h hydrolysis time and a 1/10 E/S ratio or PC_2 at 1 h hydrolysis time and a 1/50 E/S ratio. Lima bean protein hydrolysates prepared with Alcalase or pepsin-pancreatin are a potential ingredient in the production of physiologically functional foods with antihypertensive activity.

Keywords: Lima Bean; Degree of Hydrolysis; ACE Inhibition; Protein Hydrolysates; IC_{50}

1. Introduction

Hypertension is a major risk factor for stroke and other cardiovascular diseases. Among other strategies, angiotensin I-converting enzyme inhibitors (ACE-I) are used in the prevention of hypertension and congestive heart failure [1]. Studies have also been done of ACE-I in the treatment of chronic heart failure and myocardial infarction [2], as well as cancer [3,4]. ACE (peptidyldipeptide hydrolase, EC 3.4.15.1) catalyzes various reactions, two of which play key physiological roles in regulating blood pressure: conversion of inactive decapeptide angiotensin I into angiotensin II, a powerful vasoconstrictor and salt-retaining octapeptide; and inactivation of the vasodilator nonapeptide bradykinin, which is conducive to lowering blood pressure [5]. ACE-I have been shown to exhibit antihypertensive activity in spontaneously hypertensive rats and hypertensive patients [6].

Recent research has focused on ACE-I hydrolysates and peptides from animal and vegetal sources. The primary animal sources are proteins from casein, whey protein, fish protein, pig and chicken muscle, hemoglobin, blood plasma protein, gelatin [7] and egg yolks [8,9]. The primary plant sources are proteins from legume seeds such as mung bean [10], soybean [11,12], pea [13], and chickpea [14]; oilseeds such as rapeseed [15,16]; and cereal crop derivatives such as maize gluten, wheat germ and buckwheat [7]. These studies involve hydrolysate and peptide extraction with commercial enzymes (Alcalase, Flavourzyme, pepsin, pancreatin, trypsin, chymotrypsin, neutrace, etc.) at different hydrolysis times and enzyme/substrate ratios. An important variable in these studies is the concentration (mg protein/mL) of hydrolysate or peptide required to inhibit 50% of ACE-I activity under assayed conditions (IC_{50}); most enzymatic hydrolysates and peptides have values between 0.20 and 246.7 mg/mL [10]. Protein hydrolysis can be done chemically using acids or alkalis, although this oxidizes, destroys or modifies some amino acids, thus reducing protein quality [17]. Enzymatic hydrolysis is therefore preferred since it can improve the physicochemical, functional and sensory properties of native proteins

without affecting their nutritional value [18]. In addition to modifying technological functionality, enzymatic hydrolysates have also been used to reduce allergenicity, and formulate pharmaceutical products and substances for clinical applications [19,20].

Legume seeds have higher nutritional protein content (200 - 400 g/kg) than other seeds, such as cereals (70 - 140 g/kg) [21]. The high protein content of legume protein isolates or concentrates are making them increasingly important in the food industry, where they represent a promising alternative ingredient for preparing and developing new foods [22]. Germinated legumes currently account for an increasingly larger proportion of total worldwide food legume consumption because germination produces beneficial modifications in their chemical components; among other applications, they are used to produce high nutritional value flours [23]. 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 [24]. 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 [25]. *Phaseolus lunatus* is a promising source of protein hydrolysates with antihypertensive properties due to aminoacidic composition. The present study aim was to obtain hydrolysates from protein concentrates produced from ungerminated and germinated *Phaseolus lunatus* using Alcalase or pepsin-pancreatin sequential hydrolysis, and evaluate ACE-I inhibitory activity.

2. Materials and Methods

2.1. Seeds and Chemicals

Phaseolus lunatus L. seeds were purchased at a local market in Merida, Yucatan, Mexico. Any impurities were manually removed from the seeds. Flours from ungerminated and germinated seeds were prepared by cleaning the seeds, milling them in a Mycros 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 2.4 L was obtained from Novo Nordisk, Bagsvaerd, Denmark. All other chemicals were analytical grade.

2.2. Seed Germination

Seeds were washed with water, soaked in distilled water and immersed for 1 min in 20 mL sodium hypochloride 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˚C - 18˚C, with moisture levels maintained by spraying with distilled water every 8 h [26].

2.3. Protein Concentrates from Ungerminated and Germinated Seeds

Protein concentrates (PC) were extracted from the ungerminated and germinated seeds according to Chel-Guerrero *et al.* [21]. 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 ratio and then passed through 150 mesh screen to eliminate the finest fiber. Wash water was mixed with the supernatants from the initial suspension and this solution allowed 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.

2.4. Proximate Composition

Standard AOAC [27] methods were used to determine nitrogen (method 954.01); fat (920.39); ash (923.03); fiber (962.09); and moisture (925.09) contents of the flours and protein concentrates. Protein content was calculated as nitrogen (factor 6.25), and carbohydrate content was estimated as nitrogen-free extract (NFE).

2.5. Amino Acid Analysis of Protein Concentrates

Samples (2 mg) were hydrolyzed with 4 mL of 6N HCl, the solutions sealed in tubes under nitrogen and incubated in an oven at 110˚C for 24 h. Amino acids were determined after derivatization with diethyl ethoxymethylenemalonate by high-performance liquid chromatography (HPLC) [28], using D,L-g-aminobutyric acid as an internal standard. The HPLC system consisted of a Model 600E multi-system with a 484 UV-vis detector (Waters, Milford, MA) equipped with a 300 × 3.9 mm i.d. reversed-phase column (Novapack C18, 4 m; Waters). A binary gradient was used for elution at a 0.9 mL/min flow rate. The solvents used were (A) sodium acetate (25 mM) containing sodium azide (0.02% w/v) at pH 6.0,

and (B) acetonitrile. Elution was done as follows: time 0.0 - 3.0 min, linear gradient from A/B (91:9) to A/B (86/14); 3.0 - 13.0 min, elution with A/B (86/14); 13.0 - 30.0 min, linear gradient from A/B (86:14) to A/B (69/31); 30.0 - 35.0 min, elution with A/B (69:31). Eluted amino acids were detected at 280 nm and the column temperature kept at 18˚C.

2.6. Alcalase Protein Hydrolysis

Following the method of Li *et al.*, [10], protein isolate 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 2.4 L, a commercial protease preparation with endopeptidase activity produced from *Bacillus licheniformis* [14]. Its specific activity is 2.4 Anson Units (AU) per gram, one AU being the amount of enzyme which, under standard conditions, digests hemoglobin at an initial rate that generates an amount of trichloroacetic acid-soluble product that produces the same color with Folin reagent as one milliequivalent of tyrosine released per min. This enzyme is a microbial protease preparation used in the food industry to improve the functional and nutritional properties of protein preparations. For the present experiment, the specific activity of the alcalase was adjusted to 0.3 AU/g by dilution with distilled water. A 2^3 factorial design was used to analyze DH and ACE-I activity, with two replicates for both DH and ACE-I activity and four replicates of the central trial. Factors and their corresponding levels were: Factor A, protein concentrate source (ungerminated, PC_1; germinated, PC_2); Factor B, enzyme/substrate ratio (E/S) (1/50 or 1/10); and Factor C: hydrolysis time (0.5 or 2.0 h). Hydrolysis was done in a pH stat, 50 mL vessel, by adding 0.3 AU/g alcalase. 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 × g for 20 min at 4˚C (Beckman) and the supernatants freeze-dried (Labconco) for later analysis.

2.7. Pepsin-Pancreatin Protein Hydrolysis

Protein hydrolysis was done with a sequential pepsin-pancreatin system involving digestion with pepsin for half the hydrolysis time followed by incubation with pancreatin for the other half [29]. The gastric porcine pepsin (P7000, Sigma) used here has an enzymatic activity of 800 units/mg protein (one unit will produce a ΔA280 of 0.001 per min at pH 2.0 and 37˚C, measured as TCA-soluble products using hemoglobin as substrate), and the porcine pancreatin (P1500, Sigma) can hydrolyze casein more than 25 times its weight in 60 min at pH 7.5 and 40˚C. A 2^3 factorial design was used again to analyze DH and ACE-I activity, with two replicates for both DH and ACE-I activity, and four replicates of the central trial. Factors and their corresponding levels were: Factor A, PC source (PC_1, PC_2); Factor B, E/S ratio (1/50 or 1/10); and Factor C, hydrolysis time (1.0 or 3.0 h). 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.

2.8. Degree of Hydrolysis

Degree of hydrolysis (DH) was calculated by determining free amino groups through reaction with OPA reagent [30] using a serine standard. Total number of amino groups was determined in a 100% hydrolyzed sample by treatment with 6 N HCl at 110˚C for 24 h in a vacuum oven. This analysis was repeated twice.

2.9. ACE-I Activity

ACE-I activity was determined according to Hayakari *et al.* [31], with some modifications. ACE hydrolyzes HHL, yielding hippuric acid and His-Leu. This method relies on the colorimetric reaction of hippuric acid with TT in a 0.5 mL incubation mixture containing 40 μmol potassium phosphate buffer, pH 8.3; 300 μmol NaCl; 1.5 μmol HHL; 20 μL ACE (100 mU); and different amounts of hydrolysate extracts. Incubation was run for 45 min at 37˚C, and the reaction stopped by adding 1.5 mL 3% TT in dioxane, followed by 3 mL 0.2 M phosphate buffer, pH 8.3. The resulting reaction mixtures were centrifuged at 10,000 × g for 10 min, and enzymatic activity determined in the supernatant by reading absorbance at 382 nm. The IC_{50} value was determined by regression analysis of ACE inhibition (%) vs. hydrolysate protein concentration (0.1 to 0.8 mg protein/mL). Two replicates were done.

2.10. Statistical Analysis

Statistical treatment of DH and ACE-I activity results was done with an analysis of variance (ANOVA), a Duncan comparison of means and a 2^3 factorial design regression analysis. A Student *t* test (5% significance level) was applied to compare differences between means for the flour and PC chemical compositions (ungerminated and germinated seeds) [32]. All statistical analyses were done with the Statgraphics Plus version 5.0 statistical software.

3. Results and Discussion

3.1. Proximate Composition

Germination for 48 h significantly ($p < 0.05$) effected flour and PC composition (**Table 1**). This treatment reduced fat content by 16.5% (*i.e.* 1.76% - 1.47%), fiber by 15.6% (*i.e.* 5.64 - 4.76) and NFE by 4.19% (*i.e.* 58.53% - 56.08%), but increased protein content by 11.5% (*i.e.* 30.18% - 33.65%) and ash by 3.9% (*i.e.* 3.89% - 4.04%). The increase in protein and ash content after germination of *P. lunatus* seeds coincides with Dibifori *et al.* [33], who showed a 2.4% increase in protein and 0.94% increase in ash after germination of *P. lunatus* seeds for 48 h in darkness. Rodríguez *et al*. [34] report a 0.5% increase in protein content in *P. vulgaris* cv. La Granja seeds after germination for 48 h, and Bau *et al*. [35] mention increases in soybean protein content after germination. Prolonging germination time can further increase protein contents. In a study of seeds from two *P. vulgaris* cultivars (black and white seeds), protein content was found to increase (4.1% and 2.9%, respectively) after five days germination [36].

Protein concentration levels were 56.40% in PC_1 and 69.14% in PC_2. Fat content increased in both PC_1 (3.12%) and PC_2 (4.16%), probably caused by concentration of fats at the alkaline pH (11). At this pH, fat saponifies with the alkali and these (mainly polar) fats precipitate together with the protein at the isoelectric point (pH 4.5), which has been reported in protein concentrates from *Cicer arietinum* L [25] and *Canavalia ensiformis* [21]. The extent of this saponification probably depends on legume seed fatty acid composition. Germination resulted in higher protein content, probably due to synthesis of enzyme proteins for seed dicotyledon development in the mother plant and degradation of reserve nutrients (lipids and carbohydrates), both vital to the purpose of providing the energy required for protein synthesis in plant growth [35]. Protein content in both flours and PCs was similar to previously reported levels in *P. lunatus* [21,37-39]. The protein recovery percentages (*i.e.* process efficiency) for PC production were 34.30% for ungerminated seeds and 30.84% for germinated seeds, which are lower than mentioned for other seeds: 44% in faba beans [40]; 60% in lupine seeds [41]; and 46% in safflower seeds [42].

Table 1. Proximate composition of protein concentrates from ungerminated and germinated *P. lunatus* seed flours.

Component (g/100g)	F_1	F_2	PC_1	PC_2
Moisture	$(9.58)^{b}$	$(9.32)^{a}$	$(4.45)^{A}$	$(5.40)^{B}$
Protein (Nx6.25)	30.18^{a}	33.65^{b}	56.40^{A}	69.14^{B}
Fat	1.76^{b}	1.47^{a}	3.12^{A}	4.16^{B}
Fiber	5.64^{b}	4.76^{a}	1.83^{B}	0.96^{A}
Ash	3.89^{a}	4.04^{b}	3.32^{A}	3.68^{B}
NFE	58.53^{b}	56.08^{a}	35.33^{B}	22.06^{A}

$^{a\text{-}b,A\text{-}B}$Different letter superscripts in the same row indicate significant difference ($P < 0.05$); F_1: Flour from ungerminated seeds; F_2: Flour from germinated seeds; PC_1: Protein concentrate from ungerminated seeds; PC_2: Protein concentrate from germinated seeds; NFE: Nitrogen-free extract.

3.2. Amino Acids

The PCs exhibited similar amino acid profiles (**Table 2**). Both contained high proportions of Asp (12.80% and 12.47% for PC_1 and PC_2, respectively); Glu (15.30% and 16.10%), Leu (9.19% and 9.01%), Pro (7.62% and 7.68%) and Ser (7.39% and 7.26%).

Germination and the isoelectric point protein recovery process further increased levels of Glu, Gly, Ala, Met

Table 2. Amino acid composition of protein concentrates from ungerminated (PC_1) and germinated (PC_2) *P. lunatus* seed flours. (g/100g of protein).

Amino acid	PC_1	PC_2
Asp	12.80	12.47
Glu	15.30	16.10
Ser	7.39	7.26
His	3.24	3.17
Gly	4.67	4.84
Thr	4.4	4.41
Ala	6.08	6.19
Arg	4.96	5.02
Tyr	3.54	3.09
Cys	3.87	3.90
Val	4.79	4.88
Met	0.35	0.50
Phe	0.52	0.65
Ile	4.30	4.11
Leu	9.19	9.01
Lys	5.99	5.94
Pro	7.62	7.68
Trp	0.98	0.79
Amino Acid Distribution		
Hydrophobic	34.10	33.34
Neutral	20.90	21.06
Hydrophilic	45.00	45.60

and Phe, but decreased Asp, Ser, His, Tyr, Ile, Leu and Trp. In a study of germination in *P. vulgaris* for 48 h in darkness, Rodríguez *et al.* [34] report similar decreases in Lys (6.64 - 6.16 g/16g N) and Tyr (2.98 - 2.49 g/16g N), but also observed an increase in His (2.92 - 3.04 g/16g N). The PC amino acid profiles observed here are very similar to amino acid profiles reported for lima bean [39] and mung bean protein isolates [10].

3.3. Hydrolysis with Alcalase

Hydrolysis with the endoprotease Alcalase increased DH with longer hydrolysis time in both PC_1 and PC_2, the highest DH being in PC_2 (DH = 58.94%) (**Table 3**). This greater Alcalase activity in PC_2 may have resulted from partial elimination of lipids and carbohydrates during germination (**Table 2**). Another possibility is that denaturation during the concentration process produced a better molecular orientation in PC_2 than in PC_1, which would have enhanced the enzyme-substrate interaction, or that germination increased Ala, Met and Phe residues, upon which alcalase has broad specificity [19].

The variance analysis indicated that all factors and interactions influenced ($p < 0.05$) DH (Equation (1)), and the regression analysis for this response variable indicated that the results fit ($p < 0.05$) a first-order polynomial model.

$$\%\mathbf{DH} = 39.52 + 2.08A + 4.11B + 9.94\,C - 0.93AB + 01.40AC + 1.68BC + 1.28ABC \quad (1)$$

$$\mathbf{R^2} = 0.998$$

The mathematical model explaining the behavior of DH as a function of PC source (A); E/S ratio (B); hydrolysis time (C); PC source-E/S ratio (AB); PC source-hydrolysis time (AC); E/S ratio-hydrolysis time (BC); and PC source-E/S ratio-hydrolysis time (ABC) had a determination coefficient (R^2) of 0.998 (**Figure 1**, Equation (1)). This model showed E/S ratio (B) and hydrolysis time (C) to have the most positive effects on DH ($p < 0.05$), with coefficients of 4.11 and 9.94, respectively.

ACE-I activity (IC_{50}) ranged from 0.61 - 2.40 mg/mL (**Table 3**). The variance analysis showed it to be unaffected by the principal factors and two-factor interactions, but that it was affected ($p < 0.05$) by the three-factor interaction. The regression analysis showed it to fit a first-order polynomial model with an $R^2 = 0.931$. This explains the behavior of ACE-I activity as a function of PC source-E/S ratio-hydrolysis time (ABC) (Equation (2)).

$$\mathbf{IC_{50}} = 1.23 + 0.59ABC \qquad \mathbf{R^2} = 0.931 \quad (2)$$

Given that the three-factor interactions had a significant ($p < 0.05$) effect on ACE-I activity when using the three evaluated factors, the first-order, 2^3 statistical model could be optimized using higher-order models, increasing the number of factors evaluated, and evaluating the response variable as a function of the substrate and enzyme employed. From a practical standpoint, Treatment 1 (IC_{50} = 0.94 mg/mL) would be optimum because it involves lower costs (*i.e.* ungerminated seeds,

Table 3. Degree of hydrolysis (DH) and ACE inhibition (IC_{50}) in alcalase hydrolysates of protein concentrates from (a) ungerminated (PC_1) and (b) germinated (PC_2) *P. lunatus* seeds.

Treatment	Factor A Protein Concentrate Source	Factor B E/S Ratio	Factor C Hydrolysis Time (h)	DH (%)	IC_{50} (mg/mL)
1	PC_1	1/50	0.5	24.12^{a}	0.94^{d}
2	PC_2	1/50	0.5	29.91^{b}	2.40^{i}
3	PC_1	1/10	0.5	33.40^{d}	2.05^{g}
4	PC_2	1/10	0.5	30.34^{c}	0.61^{b}
5	PC_1	1/50	2.0	40.39^{f}	2.11^{h}
6	PC_2	1/50	2.0	46.67^{g}	0.87^{c}
7	PC_1	1/10	2.0	51.28^{h}	0.56^{a}
8	PC_2	1/10	2.0	58.94^{i}	1.16^{f}
9A	PC_1-PC_2 (50/50 w/w)	1/30	1.25	39.56^{e}	1.00^{e}
9B	PC_1-PC_2 (50/50 w/w)	1/30	1.25	40.07^{e}	1.02^{e}
9C	PC_1-PC_2 (50/50 w/w)	1/30	1.25	39.76^{e}	1.01^{e}
9D	PC_1-PC_2 (50/50 w/w)	1/30	1.25	39.77^{e}	1.02^{e}

[a-i]Different letter superscripts in the same column indicate significant difference ($P < 0.05$); E/S = Enzyme/substrate ratio; 9A, 9B, 9C, 9D: central trial treatments.

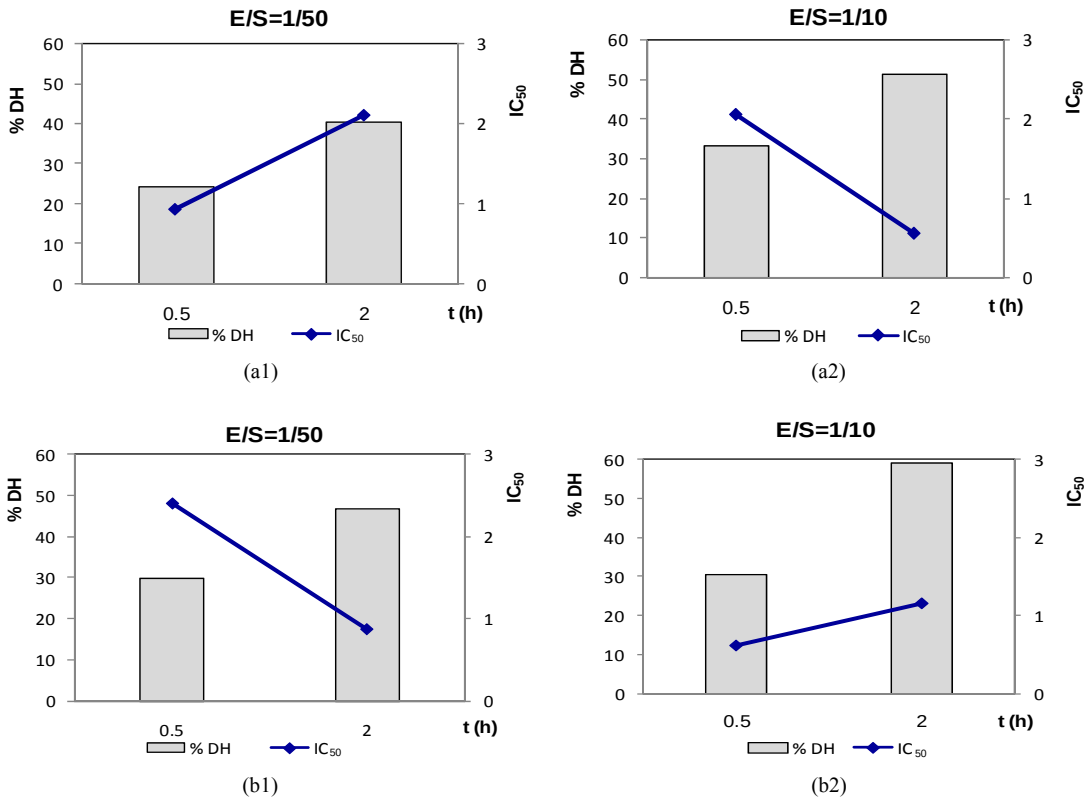

Figure 1. Degree of hydrolysis (% DH) and ACE inhibition (IC_{50}) produced by alcalase hydrolysis of protein concentrates from ungerminated ((a1) and (a2)) and germinated ((b1) and (b2)) *P. lunatus* seeds.

minimal enzyme and a shorter hydrolysis time are required), although it did not exhibit a major ACE-I effect (0.56 mg/mL).

At 0.5 h hydrolysis time and the 1/50 E/S (**Table 3**, **Figures 1(a1)**, **(b1)**), alcalase activity was highest in PC_2 (DH = 29.91%), whereas ACE-I inhibitory activity was highest in PC_1 (IC_{50} = 0.94 mg/mL). This trend inverted when the E/S ratio increased to 1/10 (**Table 3**, **Figures 1(a2)**, **(b2)**), in that alcalase activity was highest in PC_1 (33.40%) and inhibitory activity was highest in PC_2 (IC_{50} = 0.61 mg/mL). At 2 h hydrolysis time, PC_2 exhibited the highest enzymatic activity at both E/S levels (1/50: DH = 46.67%, 1/10: DH = 58.94%) (**Table 3**, **Figures 1(b1)**, **2(b2)**). In contrast, inhibitory activity was independent of PC source and E/S ratio, with the highest IC_{50} values in PC_1 at 1/10 (0.56 mg/mL) and PC_2 at 1/50 (0.87 mg/mL) (**Table 3**, **Figures 1(a1)**, **(b1)**). The inhibitory activity mathematical model and the fact that only the three-factor interaction (ABC) was significant, indicate that treatment 7 (PC_1, 1/10 E/S, 2 h hydrolysis time) produced optimum ACE-I inhibitory activity (IC_{50} = 0.56 mg/mL).

Inhibitory activity in treatments 2 (PC_2, 1/50 E/S) and 3 (PC_1, 1/10 E/S) improved with greater hydrolysis time (2.40 - 0.87 and 2.05 - 0.56 mg/mL, respectively), but declined in treatments 1 (PC_1, 1/50 E/S) and 4 (PC_2, 1/10 E/S) (0.93 - 2.11 and 0.61 - 1.16 mg/mL, respectively). This suggests that a peptide (or peptides) obtained at 0.5 h with good ACE-I activity was hydrolyzed at 2 h hydrolysis time, consequently decreasing its antihypertensive effect. In other words, there is an optimal hydrolysis point or DH above which a larger amount of ACE-I peptides are degraded than formed, lowering overall ACE-I activity. Similar behavior has been reported for protein hydrolysates from sunflower [29], chickpea [14] and fish [43]. The central trial treatment had an average DH of 39.81% and an average IC_{50} of 1.01 mg/mL, meaning that PC_1-PC_2 mixtures are not recommended in terms of their inhibitory activity. Overall, the optimum ACE-I activity range among the Alcalase hydrolysates was observed in PC_1 (IC_{50} between 0.56 - 2.11 mg/mL), probable because this enzyme can produce peptides with molecular structures which enhanced substrate ACE-I activity. However, Alcalase reacted better with PC_2, because the Alcalase cleaved more peptidic bonds which in turn generated high DH values (**Table 3**). Both the DH and inhibitory activity values for the *P. lunatus* Alcalase hydrolysates are similar to reported results for Alcalase hydrolysates from other proteins: Alaska pollack skin (IC_{50} = 0.63 mg/mL, 6 h hydrolysis time) [43]; chickpea protein isolate (IC_{50} = 0.19 mg/mL, DH = 18%, 0.5 h hydrolysis time) [44]; chickpea legumin (IC_{50} = 0.18

mg/mL, DH = 27%, 0.5 h hydrolysis time) [14]; and mung bean (IC_{50} = 0.64 mg/mL, DH = 17%, 2 h hydrolysis time) [10].

3.4. Hydrolysis with Pepsin-Pancreatin

Pepsin-pancreatin sequential hydrolysis did not hydrolyze the lima bean PCs as effectively as Alcalase, producing comparatively lower DH values (15.35% - 37.07%), although ACE-I activity was generally higher in these hydrolysates (**Table 4**, **Figure 2**). These differences in hydrolytic behavior may have originated in the hydrolysis method (e.g. enzyme type and concentration) [45], or

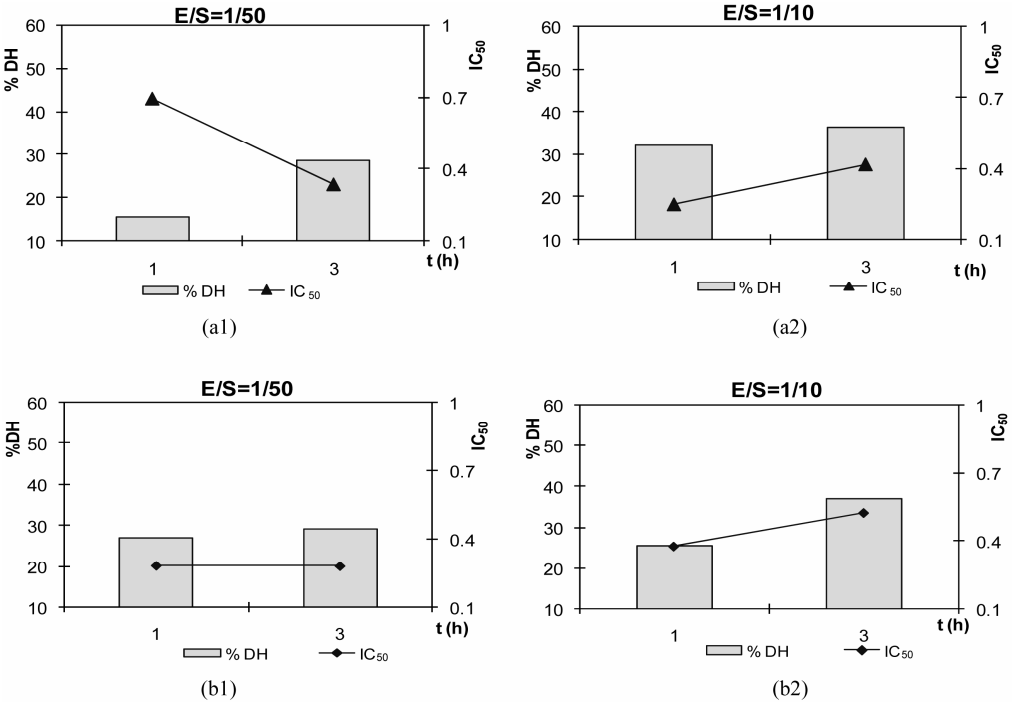

Figure 2. Degree of hydrolysis (% DH) and ACE inhibition (IC_{50}) produced by sequential pepsin-pancreatin hydrolysis of protein concentrates from ungerminated ((a1) and (a2)) and germinated ((b1) and (b2)) *P. lunatus* seeds.

Table 4. Degree of hydrolysis (DH) and ACE inhibition (IC_{50}) in pepsin-pancreatin hydrolysates of protein concentrates from (a) ungerminated (PC_1) and (b) germinated (PC_2) *P. lunatus* seeds.

Treatment	Factor A Protein Concentrate Source	Factor B E/S Ratio	Factor C Hydrolysis Time (h)	DH (%)	IC_{50} (mg/mL)
10	PC_1	1/50	1.0	15.35^{a}	0.69^{g}
11	PC_2	1/50	1.0	27.04^{c}	0.29^{b}
12	PC_1	1/10	1.0	32.16^{g}	0.25^{a}
13	PC_2	1/10	1.0	25.68^{b}	0.38^{d}
14	PC_1	1/50	3.0	28.49^{d}	0.34^{c}
15	PC_2	1/50	3.0	29.28^{e}	0.28^{b}
16	PC_1	1/10	3.0	36.28^{h}	0.42^{e}
17	PC_2	1/10	3.0	37.07^{i}	0.53^{f}
18A	PC_1-PC_2 (50/50 w/w)	1/30	2.0	29.89^{f}	0.25^{a}
18B	PC_1-PC_2 (50/50 w/w)	1/30	2.0	30.84^{f}	0.26^{a}
18C	PC_1-PC_2 (50/50 w/w)	1/30	2.0	31.04^{f}	0.25^{a}
18D	PC_1-PC_2 (50/50 w/w)	1/30	2.0	30.59^{f}	0.26^{a}

[a-h]Different letter superscripts in the same column indicate significant difference ($P < 0.05$); E/S = Enzyme/substrate ratio; 18A, 18B, 18C, 18D: central trial treatments.

the low aromatic and cationic amino acids contents (5.04% and 10.95% in PC_1, respectively; 4.53% and 10.96% in PC_2, respectively), upon which pepsin and pancreatin have broad specificity (**Table 2**) [47]. Enzymatic activity for the three evaluated enzymes differed in both experiments (0.3 AU/mL for alcalase and 4% w/v for pepsin and pancreatin) even though hydrolysis conditions (pH and temperature) were optimum for each enzyme in all treatments.

The IC_{50} values for the pepsin-pancreatin hydrolysates ranged from 0.25 to 0.69 mg/mL, with the overall higher values in PC_2 (0.28 - 0.53 mg/mL). As occurred in the alcalase hydrolysates, DH improved with increasing hydrolysis time in both PC_1 and PC_2, and inhibitory activity was independent of hydrolysis time.

The variance analysis showed that the E/S ratio, hydrolysis time, the PC source-E/S ratio interaction and the three-factor interaction (PC source-E/S ratio-hydrolysis time) influenced ($p < 0.05$) DH (Equation (3)) and the three-factor interaction (PC source-E/S ratio-hydrolysis time) influenced ($p < 0.05$) DH (Equation (3)).

$$\%\mathbf{DH} = 29.48 + 3.88B + 3.86C - 2.27AB + 2.27ABC \qquad \mathbf{R}^2 = 0.976 \tag{3}$$

The regression analysis indicated that the DH results fit ($p < 0.05$) a first-order polynomial model. This mathematical model also explained DH behavior as a function of E/S ratio (B), hydrolysis time (C), the PC source-E/S ratio (AB) interaction and the PC source-E/S ratio-hydrolysis time (ABC) interaction, with a determination coefficient of $R^2 = 0.976$. The model indicated that E/S ratio (B) and hydrolysis time (C) had most significant effect ($p < 0.05$) on DH (coefficients = 3.88 and 3.86, respectively).

None of the factors or interactions had an effect ($p > 0.05$) on ACE-I activity, that is, all of the pepsin-pancreatin treatments produced hydrolysates with ACE-I properties. Pepsin-pancreatin activity and ACE-I activity were higher (DH = 27.04%, IC_{50} = 0.29 mg/mL) in Treatment 11 (PC_2, 1 h hydrolysis time, 1/50 E/S) than in Treatment 10 (PC_1, 1 h hydrolysis time, 1/50 E/S) (**Table 4**), **Figures 2(a1)**, **(b1)**). However, enzymatic and inhibitory activities in PC_1 increased (DH = 32.16%, IC_{50} = 0.25 mg/mL) at the 1/10 E/S (**Figures 2(a2)**, **(b2)**), suggesting that both enzymes may have limited the hydrolysis reaction in PC_1 at the 1/50 E/S. By contrast, DH and inhibitory activity in PC_2 decreased at the 1/10 E/S ratio (DH, 27.04% - 25.68%; IC_{50} = 0.29 - 0.38 mg/mL) (**Figures 2(b1)**, **(b2)**). At 3 h hydrolysis time and the 1/50 E/S, DH was higher in PC_2 (29.28%) than in PC_1 (28.49%), and PC_2 exhibited improved inhibitory activity (IC_{50} = 0.28 mg/mL) (**Figures 2(a1)**, **(a2)**). At the 1/10 E/S, DH was again higher in PC_2 (37.07%) than in PC_1 (36.28%), although inhibitory activity was higher in PC_1 (0.42 mg/mL) (**Figures 2(a2)**, **(b2)**).

Optimum ACE-I activity with this hydrolysis method (0.25 mg/mL) was observed in Treatment 12. The increase from 1 to 3 h hydrolysis time increased ACE-I activity only in PC_1 at the 1/50 E/S (0.69 - 0.34 mg/mL), suggesting that this treatment generated peptides with good antihypertensive properties at 3 h hydrolysis time (**Figure 2**). Aside from a minor improvement in IC_{50} in PC_2 at 1/50 E/S with the increase from 1 to 3 h (0.29 - 0.28 mg/mL; $p > 0.05$), increased hydrolysis time generally compromised ACE-I activity, probably due to hydrolysis of some antihypertensive peptides with increased hydrolysis time. This coincides with Megías *et al* [29], who, in a study of sunflower protein hydrolysates produced with pepsin-pancreatin sequential hydrolysis, report that the highest ACE-I values were observed at DH 10% - 22%, rather than at higher DHs of 30% - 35%.

All the evaluated *P. lunatus* protein concentrate hydrolysates, those from Alcalase or pepsin-pancreatin hydrolysis, exhibited ACE-I activity, with values ranging from 0.25 to 2.40 mg/mL. The best IC_{50} value was produced using protein concentrate from ungerminated seeds sequentially hydrolyzed with pepsin-pancreatin for 1 h at a 1/10 E/S ratio. The hydrolysates from ungerminated seed flour PCs had the highest IC_{50} (0.25 - 2.11 mg/mL), although those from germinated seeds were comparable (0.28 - 2.40 mg/mL). The IC_{50} value ranges observed in the present study encompass protein hydrolysate IC_{50} values from vegetable protein sources reported in the literature: 0.18 mg/mL for maize gluten [47]; 0.18 mg/mL for chickpea legumin [14]; 1.36 mg/mL for chickpea total protein [48]; 0.23 mg/mL for rapeseed cv. León [16]; 0.30 - 1.20 mg/mL for buckwheat protein [49]; and 0.64 mg/mL for mung bean protein [10]. The IC_{50} values reported for hydrolysates from animal protein sources differ noticeably: 8 - 11.2 μg/mL for milk protein [50]; and 20 - 74.4 μg/mL for egg white protein [51].

Structure-activity correlations between different peptide ACE inhibitors indicate that ACE binding is strongly influenced by the substrate's C-terminal tripeptide sequence. The C-terminal tripeptide can interact with the three sub-sites at an active ACE site. This enzyme apparently prefers substrates or competitive inhibitors containing hydrophobic (aromatic or branched-side chain) amino acid residues at each of the three C-terminal positions. Many studies have shown that tripeptides or dipeptides with potent inhibitory activity have Trp, Tyr or Pro at their C-terminal, and branched aliphatic amino acids at the N-terminal [43]. A quantitative QSAR study of the structure-activity relationship of ACE-I peptides has also confirmed that tripeptides composed of amino acids with hydrophobicity at their C- and N-terminals have potent ACE-I activity [10]. Given that both the tested *P. lunatus*

PCs contained high proportions (33.34% - 34.10%) of hydrophobic amino acids (**Table 2**), it is probable that this action mechanism contributed to their ACE-I activity.

4. Conclusion

Protein concentrates from ungerminated and germinated *Phaseolus lunatus* seeds are good sources of ACE inhibitory peptides when hydrolyzed with the proteases Alcalase or pepsin-pancreatin. The highest ACE inhibitory activity was observed using sequential hydrolysis with pepsin-pancreatin, a potentially important fact since pepsin is the main proteolytic enzyme generated in the stomach during food digestion and pancreatin includes proteases such as trypsin, chymotrypsin and elastase, all released by the pancreas into the small intestine. In other words, the pepsin-pancreatin protein hydrolysates produced in the present study represent a set of peptides resembling those generated during digestion of *P. lunatus* proteins in the human organism. Because they are resistant to pepsin and pancreatin, these peptides could be absorbed by digestive epithelial cells in the small intestine and possibly exert an ACE inhibitory effect within the organism. Lima bean protein hydrolysates produced with pepsin-pancreatin are a potential ingredient in the production of physiologically functional foods with ACE inhibitory activity.

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