Research Article

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Angiotensin-converting enzyme-inhibitory activity in protein hydrolysates from normal and anthracnose disease-damaged *Phaseolus vulgaris* seeds

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Abstract

BACKGROUND: Bean seeds are an inexpensive source of protein. Anthracnose disease caused by the fungus *Colletotrichum lindemuthianum* results in serious losses in common bean (*Phaseolus vulgaris* L.) crops worldwide, affecting any above-ground plant part, and protein dysfunction, inducing the synthesis of proteins that allow plants to improve their stress tolerance. The aim of this study was to evaluate the use of beans damaged by anthracnose disease as a source of peptides with angiotensin-converting enzyme (ACE-I)-inhibitory activity.

RESULTS: Protein concentrates from beans spoiled by anthracnose disease and from regular beans as controls were prepared by alkaline extraction and precipitation at isolelectric pH and hydrolysed using Alcalase 2.4 L. The hydrolysates from spoiled beans had ACE-I-inhibitory activity (IC_{50} 0.0191 mg protein mL^{-1}) and were very similar to those from control beans in terms of ACE-I inhibition, peptide electrophoretic profile and kinetics of hydrolysis. Thus preparation of hydrolysates using beans affected by anthracnose disease would allow for revalorisation of this otherwise wasted product.

CONCLUSION: The present results suggest the use of spoiled bean seeds, e.g. anthracnose-damaged beans, as an alternative for the isolation of ACE-I-inhibitory peptides to be further introduced as active ingredients in functional foods.

Keywords: bioactive peptides; protein hydrolysates; anthracnose disease; ACE-I-inhibitory peptides; *Phaseolus vulgaris*

INTRODUCTION

The search for functional components in foods has become a major area of research in recent years. These components include bioactive peptides, which are small amino acid sequences that have beneficial biological activity after they are released from native proteins during gastrointestinal digestion or by previous *in vitro* protein hydrolysis.[1] Protein concentrates/isolates are used in the elaboration of many food products in order to improve their functional and/or nutritional properties. Protein hydrolysates have the additional advantage of improved functional properties as compared with the original protein isolates from which they are produced.[2,3] Recently, potential health-promoting properties have been reported in these hydrolysates, including antihypertensive, antioxidant, immunomodulatory, opioid and hypocholesterolaemic activities. Bioactive peptides with a potential antihypertensive effect due to inhibition of the angiotensin-converting enzyme (ACE-I) have been described in several animal and plant proteins.[2,4,5]

Legumes, as sessile organisms, are constantly exposed to changes in abiotic and biotic factors. The latter are the primary cause of crop losses worldwide, reducing average yields for most major crop plants by more than 50%.[6,7] Anthracnose disease is caused by the fungus *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.-Scrib. and is the most important disease of beans in rain-fed zones of Mexico. It can have devastating effects when growth of the pathogen is favoured by weather conditions.[8] The fungus attacks various plant parts, particularly pods, and reduces yields by 20–30%,[9] resulting in significant

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economic losses due to the reduction in the number of seeds per pod, mass of seeds, affecting sensory properties (such as size, texture, taste and occurrence of dark spots, which decrease their commercial value), and germination rate.[10,11] Plants rely on proteomic plasticity to remodel themselves in order to respond to abiotic and biotic stresses, resulting in the synthesis of proteins that increase stress tolerance. These include proteins involved in signalling, translation, host defence mechanisms, carbohydrate metabolism and amino acid metabolism.[7,12]

Beans affected by anthracnose cannot be used as food, although their protein content and quality are similar to those characteristic of healthy seeds. Therefore these otherwise wasted beans could be used to produce high-added-value protein hydrolysates that may be a source of bioactive peptides. In addition, it is possible that newly expressed proteins in diseased beans may be a source of novel bioactive peptides not found in healthy beans.

Antihypertensive and antioxidant activities have been found previously in protein bean concentrates after digestion with several food-grade enzyme preparations. Torruco-Uco *et al.*[13] evaluated the ACE-I-inhibitory activity of protein hydrolysates produced by enzymatic hydrolysis with Alcalase and Flavourzyme individually, obtaining IC_{50} values of 0.061 and 0.127 mg protein mL^{-1} respectively. Akillioğlu and Karakaya[14] assessed the ACE-I-inhibitory activity of common dry bean protein extracts. The effects of different heat treatment periods were investigated and the stability of the ACE-I-inhibitory activity was determined. Their results revealed that heat treatment increased the release of ACE-I-inhibitory peptides with IC_{50} values between 0.78 and 0.83 mg protein mL^{-1}, which were resistant to *in vitro* gastrointestinal digestion. Ruiz-Ruiz *et al.*[15] hydrolysed hard-to-cook bean protein concentrates using two sequential enzyme systems: Alcalase/Flavourzyme and pepsin/pancreatin. These hydrolysates were fractionated into five peptide fractions using an ultrafiltration membrane system, achieving ACE-I-inhibitory activity with IC_{50} values of 0.268–0.001 and 4.15–0.01 μg protein mL^{-1} respectively. Valdez-Ortiz *et al.*[16] hydrolysed Azufrado bean protein concentrates using Alcalase, thermolysin and pancreatin. The enzymatic treatments exerted ACE-I-inhibitory activity with IC_{50} values ranging from 0.0001 to 0.319 mg protein mL^{-1}. The differential antihypertensive potential among protein hydrolysates obtained relied mainly on the enzyme used for hydrolysis, a higher IC_{50} being acquired with pancreatin, which essentially is a combination of several digestive enzymes.

Hence the objective of the present study was to evaluate the potential of an agricultural waste product, i.e. bean seeds damaged by anthracnose disease, as a source of ACE-I-inhibitory peptides.

MATERIALS AND METHODS

Materials

Beans (*Phaseolus vulgaris* L. com. var. Jamapa) damaged by anthracnose disease (stressed seeds) were collected in 2009 at Huejutla, Hidalgo, Mexico. Control beans from an anthracnose-resistant cultivar (*P. vulgaris* L. com. var. Jamapa) were grown in 2008 at 'Santa Lucia' Experimental Station of INIFAP, Texcoco, Mexico. Both Jamapa variety samples were sown in a plot consisting of ten rows of 10 m length at spacings of 80 cm. All bean plants in the plot were naturally infected by *C. lindemuthianum*, the causal agent of anthracnose in common bean (damaged seeds). At maturity, 300 g of each sample was taken randomly from every row to form a 3 kg composite sample representative of the whole plot. Impurities were removed before whole seeds were ground.

Reagents were of analytical grade and purchased from J.T. Baker (Phillipsburg, NJ, USA), Sigma (Sigma Chemical Co., St Louis, MO, USA), Merck (Darmstadt, Germany) or Bio-Rad (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Alcalase 2.4 L was purchased from Novo Nordisk (Bagsvaerd, Denmark). This is an endoprotease preparation from *Bacillus licheniformis*, with subtilisin Carlsberg as the major enzymatic component, having a specific activity of 2.4 Anson units g^{-1}.

Proximate composition

The proximate composition of bean flours and protein concentrates was determined according to AOAC[17] procedures: moisture by method 925.09, protein by method 954.01, fat by method 920.39 and fibre by method 923.03. Total carbohydrates were estimated as nitrogen-free extract (NFE).

Preparation of Jamapa bean protein concentrates

Bean protein concentrates were prepared according to the method described by Pedroche *et al.*[4] with modifications. Whole seeds were ground to powder and passed through a 0.2 mm mesh sieve. The resulting flour was defatted by extraction with hexane for 24 h at 4 °C. Proteins were extracted by suspension of the flour in distilled water (1 : 10 w/v) adjusted to pH 9.5 with 1 mol L^{-1} NaOH and agitation for 30 min at 40 °C. The supernatant resulting from centrifugation at 5000 × *g* for 30 min was adjusted to pH 4.5 using 1 mol L^{-1} HCl in order to precipitate proteins. The protein precipitate was recovered by centrifugation at 10 000 × *g* for 30 min and lyophilised.

Enzymatic hydrolysis

Protein concentrates were hydrolysed at pH 7 and 50 °C according to the method described by Pedroche *et al.*,[4] using Alcalase 2.4 L at an enzyme/substrate ratio of 1 : 3 (w/v). After hydrolysis in a vessel equipped with a stirrer, thermometer and pH electrode, proteases were inactivated by acidification to pH 4. The resulting hydrolysates were centrifuged at 10 000 × *g* for 30 min and the supernatants were lyophilised for storage at −20 °C.

Degree of hydrolysis

Degree of hydrolysis (% DH) was determined as the percentage of soluble nitrogen in 100 g kg^{-1} trichloroacetic acid as described by Kim *et al.*[18]

ACE-I-inhibitory activity

ACE-I-inhibitory activity in protein hydrolysates was determined according to the method described by Hayakari *et al.*[19] This method is based on the colorimetric reaction of hippuric acid with 2,4,6-trichloro-*s*-triazine (TT) in a 0.5 mL incubation mixture containing 40 μmol of potassium phosphate buffer (pH 8.3), 300 μmol of sodium chloride (pH 8.3), 30 g L^{-1} HHL (hippuryl-L-histidyl-L-leucine) in potassium phosphate buffer (40 μmol, pH 8.3) and 100 mU mL^{-1} ACE-I. The mixture was incubated at 37 °C for 45 min. The reaction was terminated by adding 30 g L^{-1} TT in dioxane and 3 mL of potassium phosphate buffer (0.2 mol L^{-1}, pH 8.3). After centrifugation at 10 000 × *g* for 10 min, enzymatic activity was determined in the supernatant by measuring the absorbance at 382 nm. ACE-I-inhibitory activity was determined by regression analysis of ACE-I inhibition (%) *versus* peptide concentration (mg protein mL^{-1}) and defined as the IC_{50} value, i.e. the peptide concentration required to produce 50% ACE-I inhibition. The peptide concentrations evaluated were 0.02, 0.04, 0.06, 0.08 and 0.1 mg protein mL^{-1}.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of protein hydrolysates was performed following the method of Laemmli.[20] Protein hydrolysate samples were mixed in a 1 : 2 (v/v) ratio with buffer solution: 200 g L^{-1} SDS, 25 mL L^{-1} glycerol concentrate, 10 mL L^{-1} β-mercaptoethanol, 0.20 g L^{-1} bromophenol blue and 1 L of deionised water. The gel system consisted of 2.1 g L^{-1} SDS, 200 g L^{-1} acrylamide resolving gel (pH 8.8) and 50 g L^{-1} acrylamide stacking gel (pH 6.8). The total gel thickness was 0.75 mm, with 10 cm of resolving gel and 2 cm of stacking gel. Protein bands were stained by gel immersion in Coomassie brilliant blue R-250 (Cat. 161–0400, Bio-Rad) in a solution of 400 mL of methanol, 70 mL of acetic acid and 530 mL of deionised water.

Broad-range protein molecular weight standards (Cat. 161–0303, Bio-Rad) were used. Gel images were generated with a Gel Doc 1000 image analysis system (Bio-Rad, Richmond, CA, USA) and analysed with Molecular Analyst software (Bio-Rad).

Amino acid composition

Protein samples (2 mg) were hydrolysed in 6 mol L^{-1} HCl (4 mL) at 110 °C for 24 h in tubes sealed under nitrogen. After derivatisation with diethyl ethoxymethylenemalonate, amino acids were determined by high-performance liquid chromatography (HPLC) according to the method described by Alaiz *et al.*,[21] using D,L-α-aminobutyric acid as an internal standard and a Novapack C_{18} reverse phase column (300 mm × 3.9 mm i.d., 4 µm film thickness; Waters, Milford, MA, USA). Tryptophan was analysed by HPLC after basic hydrolysis according to Yust *et al.*[22]

Statistical analysis

Mean comparison was performed by analysis of variance and differences among means were determined by Fisher's least significant difference test at $P < 0.05$ using G-Stat Student Version 1.0 (Statpoint Technologies Inc., Warrenton, VA).

RESULTS AND DISCUSSION

Protein composition of control and damaged beans

Protein content was significantly different in control and damaged *P. vulgaris* beans, but there was no significant difference in the concentrates corresponding to control and damaged plants (Table 1). The chemical composition of a protein concentrate will depend on the source of the bean.[13–16] As compared with other seeds, these concentrates had a lower protein content than chickpea[4,23] (834 g kg^{-1}) and sunflower[24] (970 g kg^{-1}) concentrates but a higher protein content than rapeseed[21] (641 g kg^{-1}) and red lentil[23] (782 g kg^{-1}) concentrates.

As seen in Table 1, moisture, fat, fibre and NFE were significantly different ($P < 0.05$) in both control and damaged bean seeds. The fungus damage might have modified the fat content, since fat concentration was higher in damaged beans than in control beans (18.5 and 9.2 g kg^{-1} respectively); also, the content of fibre was higher. Maybe this is due to a seed–fungus interaction in which lipids and lipid metabolites influence pathogenesis and resistance mechanisms related to plant–pathogen associations.[25] Plant cell membranes also serve as reservoirs from which biologically active lipids and precursors of oxidised lipids are released as a defence response, thus showing a slight increment in fat content.[26,27] Nonetheless, fat content is among the values reported for bean flours and concentrates by Torruco-Uco *et al.*,[13] showing similar values of crude fat content in *Phaseolus lunatus* flour and protein concentrate (18.0 and 14.0 g kg^{-1} respectively) and *P. vulgaris* flour and concentrate (15.0 and 47.0 g kg^{-1} respectively), as well as for hard-to-cook bean flour and concentrate (44.1 and 27.7 g kg^{-1} respectively).[15] Moreover, Morales-de León *et al.*[28] produced protein isolates from fresh and hardened black beans exhibiting fat content values of 39.0 and 78 g kg^{-1} respectively.

Table 1. Chemical composition (g kg^{-1} dry matter) of control and damaged Jamapa bean flours and concentrates

	Flours		Concentrates	
Component	Control	Damaged	Control	Damaged
Moisture	87.7 ± 1.0^a	76.6 ± 2.0^b	64.7 ± 1.0^c	53.6 ± 1.0^d
Protein	265.4 ± 3.0^b	246.6 ± 3.0^c	800.3 ± 3.0^a	833.0 ± 3.0^a
Fat	9.2 ± 1.0^c	18.5 ± 1.0^a	6.0 ± 1.0^d	14.7 ± 1.0^b
Fibre	19.5 ± 1.0^c	43.5 ± 0.0^a	2.0 ± 0.0^d	27.2 ± 1.0^b
Ash	41.4 ± 6.0^a	45.6 ± 6.0^a	35.0 ± 5.0^b	39.2 ± 5.0^b
NFE*	576.8 ± 3.0^a	569.2 ± 2.0^b	91.5 ± 3.0^c	32.0 ± 3.0^d

Results are expressed as percent of dry matter of the mean ± SD of three determinations. Means in the same row with different letters are significantly different ($P < 0.05$).
* Nitrogen-free extract (NFE).

On the other hand, damaged beans showed a decrease in protein content. Protein concentrations in the control and damaged bean flours were 265.4 and 246.6 g kg^{-1} respectively, similar to the 260.0 g kg^{-1} reported for *P. vulgaris* flour.[29] Also for *P. vulgaris* bean flours, similar results were reported showing 268.0 and 267.5 g kg^{-1} respectively.[13,15] These results indicate that damaged beans could be utilised as a good source of protein.

Damage caused by anthracnose disease did not result in major changes in amino acid composition (Table 2). Only Arg, Val, Thr and Ile contents were higher in the damaged beans than in the control beans, probably owing to the stress stimulus, which might have induced the synthesis of proteins rich in these amino acids.[6] On the other hand, the contents of Asp + Asn, Tyr, Pro and Ser showed a significant decrease in damaged beans. Cys showed a substantial increment (0.5-fold), which is consistent with the role that this sulfur-containing amino acid has in seed protection against fungi.[30] As compared with FAO requirements, the only limiting amino acids in the flours and concentrates were Met and Cys.

SDS-PAGE analysis of both control and damaged beans (Fig. 1) showed at least ten bands corresponding to proteins with apparent molecular weights ranging from 15 to 200 kDa. The major band corresponds to phaseolin (46 kDa subunit), which is the most abundant protein in *P. vulgaris*. Other bands correspond to phytohaemagglutinins PHA-E (32 kDa) and PHA-L (22 kDa), α-amylase inhibitor (18 kDa) and α-amilase β subunit PHA-I β subunit (16 kDa).[29,31,32]

Damage by anthracnose disease did not appear to cause any change in the electrophoretic profile.

Production of bean protein hydrolysates

Treatment of the protein concentrates with Alcalase resulted in very similar hydrolysis curves for both the control and anthracnose concentrates. DH values increased to 32–35% after 30 min, and further incubation did not result in any significant increase in DH (Fig. 2).

Table 2. Amino acid composition (g kg^{-1}) of control and damaged Jamapa bean flours and protein concentrates

Amino acid	Control bean flour	Damaged bean flour	Control bean protein concentrate	Damaged bean protein concentrate	FAO[a]
Asp + Asn	128.0 ± 2.0^{a}	110.0 ± 1.0^{b}	113.0 ± 1.0^{b}	95.0 ± 1.0^{c}	
Glu + Gln	165.0 ± 1.0^{b,a}	165.0 ± 0.0^{b}	171.0 ± 1.0^{a}	171.0 ± 2.0^{a}	
Ser	75.0 ± 1.0^{a}	68.0 ± 1.0^{b}	71.0 ± 0.0^{b}	64.0 ± 0.0^{c}	
His	31.0 ± 1.0^{b}	31.0 ± 0.0^{b}	35.0 ± 0.0^{a}	35.0 ± 0.0^{a}	19.0
Gly	48.0 ± 2.0^{a}	49.0 ± 0.0^{a}	43.0 ± 0.0^{b}	44.0 ± 0.0^{b}	
Thr	46.0 ± 1.0^{b}	53.0 ± 0.0^{a}	42.0 ± 0.0^{c}	49.0 ± 0.0^{b}	34.0
Arg	72.0 ± 0.0^{b}	80.0 ± 0.0^{a}	60.0 ± 0.0^{d}	68.0 ± 0.0^{c}	
Ala	48.0 ± 2.0^{a}	47.0 ± 1.0^{a}	41.0 ± 0.0^{b}	40.0 ± 0.0^{b}	
Pro	28.0 ± 1.0^{a}	21.0 ± 1.0^{b}	20.0 ± 0.0^{b}	13.0 ± 0.0^{c}	
Tyr	37.0 ± 1.0^{b}	28.0 ± 0.0^{d}	41.0 ± 0.0^{a}	32.0 ± 0.0^{c}	
Val	45.0 ± 1.0^{c}	54.0 ± 0.0^{b}	57.0 ± 0.0^{b}	66.0 ± 0.0^{a}	35.0
Met	8.0 ± 1.0^{a}	6.0 ± 0.0^{a}	8.0 ± 0.0^{a}	6.0 ± 0.0^{a}	25.0^{b}
Cys	4.0 ± 1.0^{b}	8.0 ± 0.0^{a}	3.0 ± 1.0^{b}	7.0 ± 2.0^{a}	
Ile	40.0 ± 2.0^{d}	48.0 ± 0.0^{c}	55.0 ± 0.0^{b}	63.0 ± 0.0^{a}	28.0
Trp	9.0 ± 0.0^{a}	9.0 ± 0.0^{a}	2.0 ± 0.0^{b}	2.0 ± 0.0^{b}	11.0
Leu	85.0 ± 2.0^{b}	87.0 ± 0.0^{b}	99.0 ± 1.0^{a}	101.0 ± 1.0^{a}	66.0
Phe	61.0 ± 2.0^{cb}	64.0 ± 1.0^{b}	68.0 ± 4.0^{a}	71.0 ± 3.0^{a}	63.0^{c}
Lys	70.0 ± 1.0^{b}	72.0 ± 1.0^{b}	74.0 ± 1.0^{a}	76.0 ± 1.0^{a}	58.0
Amino acid distribution					
Hydrophobic	324.0 ± 2.0^{d}	336.0 ± 2.0^{c}	350.0 ± 2.0^{b}	362.0 ± 1.0^{a}	
Neutral	210.0 ± 1.0^{a}	206.0 ± 1.0^{a}	200.0 ± 2.0^{b}	196.0 ± 3.0^{b}	
Hydrophilic	466.0 ± 2.0^{a}	458.0 ± 3.0^{b}	450.0 ± 2.0^{c}	442.0 ± 3.0^{d}	

Data are mean ± standard error of at least three replicates.
Values in the same row with different letters are significantly different ($P < 0.05$).
[a] FAO/WHO protein quality evaluation 1985. (FAO/WHO/ONU. Energy and protein requirements. Reports of a joint meeting. WHO, Geneva, 1985, Technical report series N.° 724).
[b] Met + Cys.
[c] Phe + Tyr.

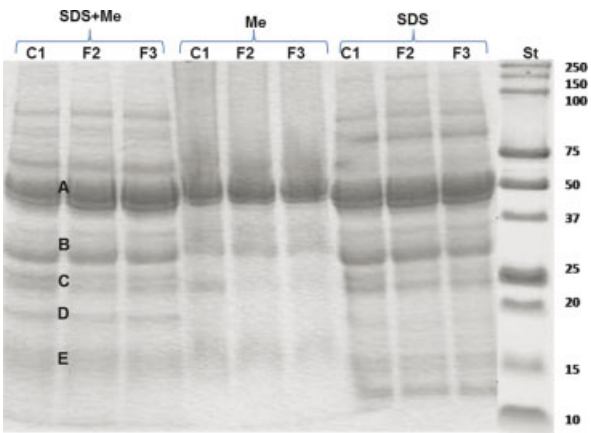

Figure 1. SDS-PAGE of control protein concentrate (C1) and flour (F3) and flour from beans damaged by anthracnose disease (F2): A, subunit phaseolin; B, PHA-E; C, PHA-L; D, α-amylase inhibitor; E, α-amilase β subunit PHA-I β subunit. Reducing and denaturating (SDS + Me), only reducing (Me) and only denaturing (SDS) conditions were used for each sample. Lane St: MW standards (kDa).

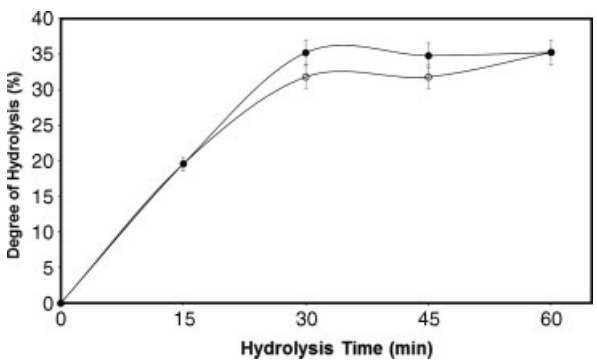

Figure 2. Hydrolysis of control (●) and damaged (○) beans by treatment with Alcalase. Data are mean ± standard error of at least three replicates.

DH values for the *P. vulgaris* hydrolysates obtained in the present study using Alcalase were slightly different from the 49.48% at 30 min reported for *P. vulgaris* protein concentrate[13] and the 22% at 10 h reported for mung bean (*P. vulgaris* L.) protein hydrolysate[33] obtained with the same enzyme. Similarly, hard-to-cook bean protein hydrolysates were produced sequentially with Alcalase/Flavourzyme and pepsin/pancreatin showing DH values of 43.01 and 26.15% respectively at 90 min for both systems.[15] This could be due to differences among hydrolysis parameters such as enzyme/substrate ratio, hydrolysis time and substrate concentration and could also be explained by the differential protein profile found in seed storage proteins of legumes;[14,16,23] possibly, secondary and tertiary structures from the proteins differ

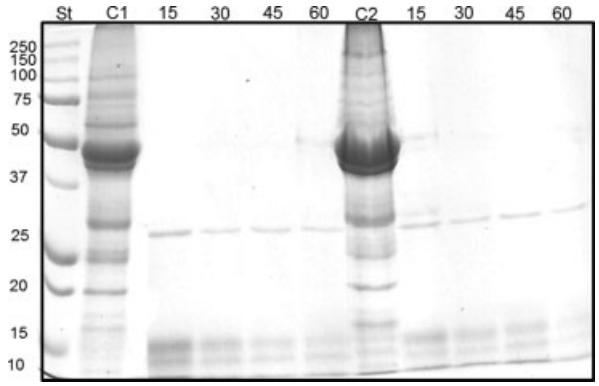

Figure 3. SDS-PAGE of control and damaged protein concentrates and hydrolysates at different reaction times after treatment with Alcalase. Concentrates from control (C1) and damaged (C2) beans were treated with Alcalase for 15–60 min. Lane St: MW standards (kDa).

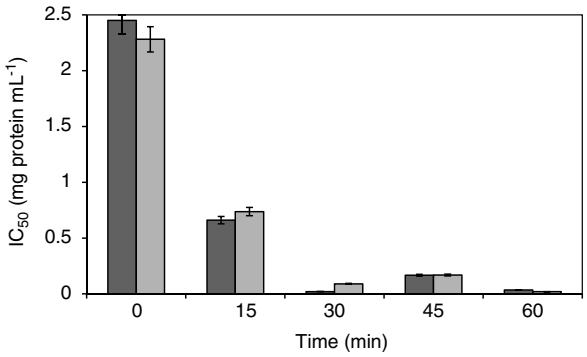

Figure 4. ACE-I-inhibitory activity in hydrolysates produced by treatment of control (□) and damaged (■A) bean protein concentrates with Alcalase. Data are mean ± standard error of three replicates.

among themselves, thus making the protein concentrates more or less susceptible to proteolysis.[14,16,18,23]

Amino acid analysis showed that *P. vulgaris* protein concentrates comprise a high proportion of hydrophobic amino acids (Table 2), which may represent a good target for Alcalase hydrolysis. Alcalase hydrolyses peptide bonds with wide-range specificity, releasing peptides with hydrophobic amino acids such as Tyr, Leu, Phe, Trp, Val, Ile and Met at their C-terminal,[34] so anthracnose-damaged beans are a good protein source of such bioactive peptides upon hydrolysis by Alcalase, as verified by this research.

A quantitative structure–activity relationship study of ACE-inhibitory peptides has also confirmed that tripeptides composed of amino acids with strong hydrophobicity at their C- and N-terminal have potent ACE-inhibitory activity.[33] Amino acid analysis of Jamapa bean protein flours and concentrates showed that they contain a high proportion of hydrophobic amino acids (Table 2). Of many ACE-inhibitory peptides identified from different food sources,[4,13–16,18] structure–activity studies indicated that C-terminal tripeptide residues play a predominant role in competitive binding to the active site of ACE.[35] It has been reported that this enzyme prefers substrates or inhibitors containing hydrophobic (aromatic or branched side chain) amino acid residues at each of the three C-terminal positions.[35] The most effective ACE-inhibitory peptides identified contain Tyr, Phe, Trp and/or Pro at the C-terminal. Also, Ruiz *et al*.[36] have suggested that Leu may contribute significantly to increase ACE-inhibitory potential, an important amount of this amino acid being shown for both samples.

SDS-PAGE analysis of the hydrolysates (Fig. 3) corroborated that treatment with Alcalase was effective, leaving only low-molecular-weight (low-MW) peptides even after treatment for only 15 min, in addition to a protein band at about 29 kDa that appeared to be relatively resistant to hydrolysis, probably corresponding to a protease inhibitor. In contrast, Torruco-Uco *et al*.[13] hydrolysed a *P. vulgaris* concentrate with Alcalase for 90 min, observing low-MW bands between 26.5 and 13.0 kDa that disappeared at 90 min of hydrolysis, although a band with an approximate MW of 27–29 kDa was also observed until 75 min, which decreased in intensity at 90 min of hydrolysis.

ACE-I-Inhibitory activity

ACE-I-inhibitory activity was assayed in the hydrolysates and IC_{50} was calculated (Fig. 4). The inhibitory activity resembled the hydrolysis curves (Fig. 2), showing an IC_{50} value of 0.7 mg protein mL^{-1} after treatment with Alcalase for 15 min, and IC_{50} values lower than 0.2 mg protein mL^{-1} after treatment for 60 min or longer, with no significant difference between hydrolysates (Fig. 4).

These values are similar to the IC_{50} value of 0.18 mg protein mL^{-1} previously reported by Yust *et al*.[22] for chickpea legumin hydrolysed with Alcalase. It is of interest that the highest DH corresponded to the highest ACE-I-inhibitory activity for both damaged and control Jamapa bean hydrolysates, while after 45 min of hydrolysis a decrease in ACE-I-inhibitory activity was observed (IC_{50}0.167 and 0.169 mg protein mL^{-1} respectively). This may be due to the ACE-I-inhibitory peptides being hydrolysed to produce inactive small peptides or amino acids upon further increase in hydrolysis time. Pedroche *et al*.[4] reported that chickpea protein ACE-I-inhibitory fragments became a target of the enzyme and were also hydrolysed with an increase in hydrolysis time.

Li *et al*.[33] hydrolysed mung bean protein isolates with Alcalase and measured the ACE-I-inhibitory activity. The non-hydrolysed protein showed no inhibitory activity, while the highest ACE-I-inhibitory activity (IC_{50} 0.64 mg protein mL^{-1}) was found in the hydrolysate obtained with Alcalase after 2 h of hydrolysis. Valdez-Ortiz *et al*.[16] hydrolysed protein concentrates from three cultivars of Azufrado bean using Alcalase, thermolysin and pancreatin for 2 h. The treatments with the lowest values of IC_{50} were the Alcalase protein hydrolysates for all three cultivars (0.0001, 0.0004 and 0.013 mg protein mL^{-1}). Ruiz-Ruiz *et al*.[15] obtained protein isolates from hard-to-cook beans and hydrolysed them with a sequential enzymatic system. The hydrolysates produced were fractionated into five peptide fractions (>10, 5–10, 3–5, 1–3 and <1 kDa) using an ultrafiltration membrane system, with higher IC_{50} values (0.001 µg protein mL^{-1}) being shown for the <1 kDa fraction. Akillioğlu and Karakaya[14] evaluated the ACE-I-inhibitory activity of common dry beans, dry pinto beans and green lentils and the effects of different heat treatment periods, observing that 30 min of heat treatment caused a decrease in ACE-I-inhibitory activity of the legume samples studied, although 50 min of heat treatment resulted in an increase in ACE-I-inhibitory activity of the samples. Nonetheless, the ACE-I-inhibitory activity of the three legume samples increased following *in vitro* gastrointestinal digestion (0.78–0.83, 0.15–0.69 and 0.008–0.89 mg protein mL^{-1} respectively).

The stress stimulus produced in the damaged bean seeds did not modify the ACE-I-inhibitory activity of peptides released by enzymatic hydrolysis, so anthracnose-damaged beans could be used as a potential source of bioactive peptides with diverse bioactivities.

CONCLUSIONS

Damage by anthracnose disease to *P. vulgaris* var. Jamapa beans does not affect the ACE-I-inhibitory activity that can be released by treatment of protein concentrates with Alcalase, resulting in IC_{50} values as low as 0.0191 mg protein mL^{-1}. Thus these beans are a good raw material for enzyme-mediated production of ACE-I-inhibitory peptides, which could greatly increase the value of this otherwise wasted product. Further isolation and identification of specific peptides with ACE-I-inhibitory activity, elucidation of the relationship between peptide structure and activity, and its antihypertensive effect *in vivo* await future study.

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