Angiotensin I-converting enzyme-inhibitory peptide fractions from albumin 1 and globulin as obtained of amaranth grain

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ABSTRACT

A number of biopeptides promoting health benefits have been isolated from food-protein hydrolysates and can be released during enzymatic digestion. Antihypertensive peptides can be part of protein fractions from amaranth grain. The objective of this work was to obtain ACE-inhibitory peptide fractions from albumin 1 and the globulin of amaranth (*Amaranthus hypochondriacus*) grain. Albumin 1 and globulin were hydrolysed with alcalase; hydrolysis was monitored by proteolytic degradation and by ACE-inhibitory activity. The highest ACE-inhibitory activity was 40% and 35% as obtained after 18 and 15 h hydrolysis for albumin 1 and globulin, respectively. Further separation and purification of the ACE-inhibitory peptide fractions were carried out by gel filtration and C_{18} RP–HPLC. The IC$_{50}$ was 0.35 ± 0.02 mg/ml for albumin 1 peptide fraction and 0.15 ± 0.03 mg/ml for globulin peptide fraction. Albumin 1 peptide fraction showed an competitive mode of ACE inhibition, whereas the globulin peptide fraction was competitive. The globulin peptide fraction may have one of the most active naturally-occurring ACE-inhibitory peptides.

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1. Introduction

Grain amaranth is a pseudo cereal with a high protein content (17–19% of grain dry weight) when compared to more traditional crops that have an average of ≈10% protein. It is rich in essential amino acids such as lysine, tryptophan, and sulphur-containing amino acids. In Amaranthus, 50% of the total seed proteins at maturity are globulin and albumin (Paredes-López, Mora-Escobedo, & Ordorica-Falomir, 1988; Soriano-Santos, Iwabuchi, & Fujimoto, 1992). Amaranth proteins are mostly storage proteins with legumin-like features. They comprise globulin (11S-globulin and globulin-*p*) and glutelins, which present different solubility in aqueous solvents. The 11S-globulin is extracted by treating the flour with a saline, neutral buffer (Γ = 0.5 M) and globulin-*p* (formerly named albumin 2 by Konishi, Horikawa, Oku, Azumaya, & Nakatani, 1991) is extracted with water after the saline treatment of the flour. In addition, glutelins, storage-aggregated proteins, are only soluble in alkaline or acidic media. Konishi et al. (1991) described two types of albumin in amaranth: albumin 1, removed with water and/or saline solutions; and albumin 2, extracted with water after the flour had been treated with saline solutions in order to remove albumin 1 and globulins. Albumin 2, recently called globulin-*p*, is scarce. It is formed by several polypeptides subunits, very similar to amarantin except for the presence of the 54 kDa subunit and its tendency to polymerise. It has previously been reported that globulin belongs mostly to the 11S type, which also has amarantin. A minor fraction of the 7S leguminous type has also been identified (Romero-Zepeda & Paredes-López, 1996; Segura-Nieto, Barba de la Rosa, & Paredes-López, 1994, chap. 5). This amarantin has already been purified, crystallized and characterised by means of X-ray diffraction. Recently lunasin, known as a bioactive peptide, has also been found in amaranth; it is a 43 amino acid peptide whose cancer preventive properties have been demonstrated in biological models. Additionally, it has been predicted that amaranth may be a source of peptides with 11 different potential biological activities such as antihypertensive, protease inhibitor, opioid, immune-modulating, antithrombotic, antioxidant, amongst others. Amaranth globulins and glutelins may have the highest antihypertensive peptides in their amino acid sequences (Silva-Sánchez et al., 2008).

In recent years, peptides from partial enzymatic hydrolysates of food proteins have received a greater deal of attention from food scientists than ever before. Many biological peptides promoting health benefits have been classified and identified from food-protein hydrolysates. These peptides are inactive within the sequence

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of the parent protein, but can be released during enzymatic digestion or food processing. Great accomplishments have already been achieved and some of these peptides, such as casein phosphopeptide and antihypertensive peptides, have already been marketed in Japan (Wu & Ding, 2002).

Angiotensin I-converting enzyme (ACE; peptidylpeptide hydrolase, EC 3.4.15.1) plays an important role in the regulation of blood pressure as well as in cardiovascular function. ACE generates the powerful vasoconstrictor angiotensin II that removes the C-terminal dipeptide from the lysate of the precursor decapeptide angiotensin I. It also inactivates the vasodilator bradykinin (Skeggs, Marsh, Kahn, & Shumway, 1954). Thus, high levels of ACE can lead to increased vasoconstriction and, consequently, the development of high blood pressure with its accompanying pathological symptoms. Because hypertensive patients often need life-long medical treatment, interest has been focused on the isolation and identification of ACE-inhibitory peptides which may be obtained from new and varied food sources. Therefore, the objective of this work was to obtain ACE-inhibitory peptide fractions produced by alcalase treatment of albumin 1 and globulin of amaranth (*Amaranthus hypochondriacus*) grain, which is a new food source.

2. Materials and methods

2.1. Raw material

Amaranth grain (*A. hypochondriacus*) was harvested at Tulyehualco, Mexico City, Mexico during autumn. The flour was obtained by grinding the whole grain through a Udy mill (Udy Corporation, Fort Collins, Co, USA); it was then extracted 3 times with acetone (5 ml/g) with continuous stirring at room temperature for 16 h, washed with diethyl ether (400 ml) and air-dried (at room temperature). The defatted meal was again sieved through a 60-mesh screen and stored at 5 °C until further analysis.

2.2. Chemical analysis

All analyses throughout this research were performed in triplicate. Proximate analysis was carried out following the AOAC (2000) standard procedures; it included moisture, ash, crude protein, ether extract and crude fibre; carbohydrate concentration was calculated by difference.

2.3. Protein fractions extraction

The amaranth grain protein extraction was conducted according to the method reported by Soriano-Santos et al. (1992). Defatted amaranth flour (50 g) was mixed with 300 ml 0.04 M Na_2SO_4 + 20 mM β-mercaptoethanol and stirred for 30 min, centrifuged for 20 min at 13000g; the supernatant was mixed with 50%, 70% and 100% (sat.) $(NH_4)_2SO_4$ and dialysed for 24 h with four changes of distilled water; this method separated albumin 1 from globulin.

Albumin 1 and globulin extraction were also carried out in order to compare them to the previous procedure, according to the method reported by Drzewiecki et al. (2003). Defatted amaranth flour was mixed with 0.5 M NaCl (1:10) for 30 min at 4 °C. The slurry was centrifuged at 9000g for 20 min. The supernatants were dialysed (Mw cutoff 6000) for 3 days against deionized water at 4 °C. The content of dialysis tubes was centrifuged at 9000g for 20 min. The supernatant was albumin 1 and the pellet was globulin fraction.

All extracts were lyophilised and stored at 5 °C until analysis. The albumin 1 and globulin yield were quantified in each method by evaluating nitrogen content according to Kjeldahl's method (AOAC, 2000) and protein content according to Bradford's (1976). Protein content for each fraction was expressed as g per 100 g defatted flour. Globulin/albumin 1 ratio (G/A) for each extraction

method was calculated by dividing the protein content of every fraction.

2.4. Protein hydrolysis

The obtained albumin 1 and globulin fractions were hydrolysed following the method reported by Surovtsev et al. (2001) with modifications; 320 μL 0.5 M phosphate buffer (pH 7.4) were added to 600 μl protein solution (5 mg/ml). The solution was incubated for 5 min at 50 °C; 80 μl alcalase solution (165 mUA) in 0.5 M phosphate buffer, pH 7.4, were added to each test tube to 0.8 UA/g protein final concentration. The reaction was stopped by adding 100 μl phenylmethylsulfonyl fluoride solution in ethanol (2 mg/ml) for 1 to 48 h.

2.5. Degree of hydrolysis (DH)

DH was analysed for free amino groups, according to the method described by Adler-Nissen (1979). One ml 0.5 M phosphate buffer, pH 8.2 and 1 ml 2,4,6-trinitrobenzenesulfonic acid solution in water (1 mg/ml) was added to 125 μl hydrolysed albumin 1 or globulin at 1 to 48 h. The reaction mixture was incubated for 1 h at 50 °C in the dark as light accelerates the reaction. Trinitrophenylation was stopped by the addition of 2 ml 0.1 N HCl; the mixture was left at room temperature for 30 min; absorbance was read at 340 nm against water. The blank was prepared in the same manner. DH was calculated as follows:

$$\%DH = (h/h_{tot}) \times 100$$

where DH, disrupted peptide bonds (h)/total peptide bonds per weight unit (h_{tot}). L-Leucin was used as a standard. The analysis was carried out in triplicate.

2.6. ACE-inhibitory activity assay

ACE-inhibitory activity was analysed according to a modification of the method described by Hayakari, Kondo, and Izumi (1978) method; hippuryl-L-histidyl-L-leucine (HHL) was used as the substrate. ACE hydrolyses HHL to hippurate (HA), which reacts with cyanuric chloride to yield a chromogen quantified by absorbance at 382 nm. Two solutions were separately prepared: (A) hydrolysed centrifuged albumin 1 or globulin (50 μl); 50 μl 40 μM potassium phosphate buffer, pH 8.3, and 100 μl 0.2 M HHL were heated at 37 °C; (B) 150 μl 0.2 M boric acid–NaCl, pH 8.3 + 100 μl 300 μM NaCl + 50 μl ACE (165 mU) in phosphate buffer, pH 7.4. The mixture was also heated at 37 °C for 15 min. Both solutions were combined and incubated at 37 °C for 30 min. The reaction was stopped by placing the mixture in a boiling-water bath for 10 min. One ml 0.2 M potassium phosphate buffer, pH 8.3, followed by 1.5 ml 3% cyanuric acid (2,4,6-trichloro-S-triazine) in dioxane were added to the reaction mixture, and vigorously mixed in a vortex until the solution became transparent. The solution absorbance was measured at 382 nm. The IC_{50} value was defined as the peptide concentration (mg protein/ml) necessary to inhibit 50% ACE activity; it was calculated by an ACE inhibition (%) vs. log peptide concentration (mg/ml) linear regression (Li, Le, Liu, & Shi, 2005). Captopril and ACE inhibitor (pGlu–Trp–Pro–Arg–Pro–Gln–Ile–Pro–Pro) were used as standards.

2.7. Gel filtration chromatography

Previous to alcalase hydrolysis, albumin 1 and globulin were characterised by gel filtration. One hundred milligrams of the hydrolysed protein fraction was dissolved in 5 ml 32.5 mM K_2HPO_4–2.6 mM KH_2PO_4, pH 7.5 (buffer A: 0.4 M NaCl + 20 mM 2-mercaptoethanol, $\Gamma = 0.5$) (Konishi, Fumita, Ikeda, Okuno, &

Fuwa, 1985). Albumin 1 and globulin were applied to a 1.4 × 29 cm, id Sephadex G-200 column (Pharmacia, Uppsala, Sweden). The column was previously equilibrated with buffer A. One-millilitre fractions, 0.4 ml/min flow rate, were collected at room temperature and read at 280 nm. A low range molecular weight marker (Sigma–Aldrich, St. Louis, Mo., USA) was used (tyroglobulin, 670 kDa; gamma globulin, (158 kDa; ovoalbumin, 44 kDa; myoglobin, 17 kDa; vitamin B-12, 1.35 kDa). After alcalase hydrolysis, albumin 1 and globulin were further fractionated and applied to the same column to observe Mr changes undergone after enzymatic treatment. The fractions from two replicate chromatography runs were pooled and then lyophilised and stored at −25 °C until their application to a Sephadex G-15 column (Pharmacia, Uppsala, Sweden, 1.4 × 29 cm), and eluted under the same experimental conditions described above; this method was used to separate the peptide fractions with ACE-inhibitory activity, but detected at 214 nm. In this step, the Ultra-low range molecular weight marker (Sigma–Aldrich, St. Louis, Mo., USA) was used (triose phosphate isomerase 26.6 kDa; myoglobin 17 kDa; α-lactalbumin 14.2 kDa; aprotinin 6.5 kDa; insulin 3.5 kDa and bradykinin 1.06 kDa). Peptide fractions were collected (AI.1.1, AI.1.2, G1.1, G1.2, G2.1 and G2.2), and corresponding fractions from chromatography runs were pooled, until a sufficient amount was gathered to assess IC_{50}. Those fractions were lyophilised and stored at −25 °C until use.

2.8. Electrophoresis (sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE))

Albumin 1 and globulin SDS–PAGE profiles were carried out according to the method proposed by Laemmli (1970), using 15.5% (w/v polyacrylamide) separating gel and stacking gel (4% w/v polyacrylamide). All gels were run in mini-slabs (Bio-Rad, Mini Protean II Model, Hercules, CA, USA) at 200 volts. Protein samples were prepared by mixing 3 vol of the respective chromatographic fraction with 1 vol of concentrated sample buffer. Protein bands were visualised with Coomassie brilliant blue R-250. The molecular weight of polypeptides was calculated using the MW–SDS-70 protein standard (Sigma–Aldrich, St. Louis, Mo., USA): ovoalbumin 45 kDa; carbonic anhydrase 34.7 kDa; trypsin inhibitor 24 kDa; myoglobin 18.4 kDa; lactalbumin 14.3 kDa .

2.9. Reversed-phase high performance liquid chromatography (RP–HPLC)

To purify antihypertensive peptide fractions, those with high IC_{50} values from Sephadex G-15 were re-dissolved in distilled, deionized water; 20 μl of each fraction was loaded onto a Nucleosil 100 C_{18} RP (4.6 × 250 mm, i.d.; 5 μm particle size, Alltech, Deerfield, IL, USA) that was equilibrated in 0.1% (v/v) trifluoroacetic acid (TFA) in water and connected to a HPLC system (Thermo Separation Products, Cincinnati, Ohio, USA). Albumin 1 and globulin peptide fractions were eluted using a gradient of acetonitrile + 0.1% (v/v) of TFA from 0 to 30% (v/v) over 60 min at a flow rate of 2 ml/min; elution was monitored at 214 nm. Peptide fractions were collected (AI.1.2.1, AI.1.2.2, AI.1.2.3, G2.2.1, G2.2.2 and G2.2.3) and their corresponding fractions of replicate chromatography runs were pooled until a sufficient amount was obtained to assess IC_{50}. Those fractions were lyophilised and stored at −25 °C until use.

2.10. Inhibitory kinetics of amaranth ACE-inhibitory peptide fractions

The peptide fractions with the highest IC_{50} from albumin 1 and globulin were analysed for an inhibition pattern. Various substrate (HHL) concentrations ranging from 100 to 400 mM, were incu-

bated with ACE solution in the presence of the appropriate concentration (3–10 mM) of albumin 1 peptide fraction (AI.1.2.2) and globulin peptide fraction (G2.2.2) purified by RP–HPLC. The initial reaction velocities were determined from the formation of product HA over time. The Km and V values for the reaction at different concentrations were determined according to Lineweaver-Burk plots.

3. Results and discussion

3.1. Chemical analysis and albumin 1 and globulin extraction

Table 1 shows the proximate analysis of the A. hypochondriacus grain and defatted flour used in this study. The present results show that crude protein (N × 5.85) was not appreciably concentrated when the flour was defatted with acetone. On the contrary, ash and carbohydrates increased because of this process. Even though this protein concentration is not very significant (17.25 ± 0.18 g/100 g dry weight), the defatted amaranth flour was used to isolate albumin 1 and globulin to obtain peptide fractions by a further alcalase hydrolysis treatment. For further research, a different process, such as the pneumatic classification system should be used to obtain a protein concentrate before obtaining peptides, if these are to be applied in the food industry.

Even though general agreement has not been achieved as to which amaranth fraction is the largest, several researchers have considered that albumin appears to be the largest in amaranth grain (Segura-Nieto, Barba de la Rosa, & Paredes-López, 1994, chap. 5; Soriano-Santos et al., 1992) followed by globulins (Konishi et al., 1985) and glutelins (Paredes-López et al., 1988). In this work, albumin and globulin were studied with regard to their ACE-inhibitory potential. When comparing two different extraction methods to obtain albumin 1 and globulin, with their globulin/albumin (G/A) ratio (Table 1), the G/A was 0.2, regardless of the method being used. However, when 29.3% NaCl, which corresponds to 0.5 M, was used to solubilise these proteins, a larger amount of non-protein nitrogen was dissolved than when 5% Na_2SO_4 (0.04 M) was used. G/A values ranged from 0.3 to 2.1 (Konishi et al., 1985; Segura-Nieto, Barba de la Rosa, & Paredes-López, 1994, chap. 5; Soriano-Santos et al., 1992), which depend on several factors such as the extraction method, the ionic strength of solution used, the pH,

Table 1
Proximal analyses of both whole grain and defatted flour from *Amaranthus hypochondriacus* grain as well as the G/A[a] ratio by using two different extraction methods.

	Whole grain (g/100 g)	Defatted flour (g/100 g)	
Moisture	10.9 ± 0.11[b]	10.9 ± 0.12	
Crude protein[c]	14.1 ± 0.17	15.4 ± 0.18	
Ether extract	7.14 ± 0.14	0.22 ± 0.00	
Crude fibre	4.56 ± 0.04	4.91 ± 0.08	
Ash	2.85 ± 0.04	3.27 ± 0.01	
Carbohydrates[d]	60.5 ± 0.02	65.4 ± 0.06	

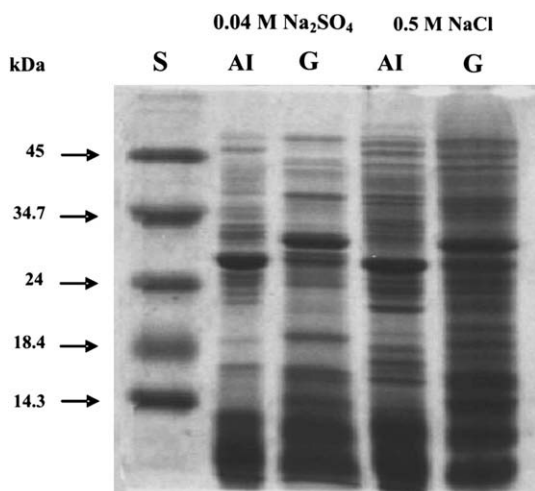|	Extraction method			
	0.04 M Na_2SO_4		0.5 M NaCl	
	Albumin 1	Globulin	Albumin 1	Globulin
	g/100 g defatted amaranth flour			
Crude protein	1.54 ± 0.12[b]	0.35 ± 0.02	4.72 ± 0.33	0.61 ± 0.04
Protein[e]	1.45 ± 0.17	0.32 ± 0.01	1.76 ± 0.12	0.36 ± 0.01
G/A ratio	0.22		0.20	

[a] Globulin/albumin ratio.
[b] Data are average ± standard deviation of triplicate determinations.
[c] Nitrogen measured by the method of Kjeldahl (%N × 5.87).
[d] Calculated by difference.
[e] Protein measured by the method of Bradford (1976).

Fig. 1. SDS–PAGE profile by using two different extraction methods for albumin 1 (AI) and globulin (G) from the *Amaranthus hypochondriacus* grain. S, standard proteins; kDa, molecular weight of the standard proteins.

etc. In addition, the electrophoretic profiles of albumin 1 and globulin, (Fig. 1) were very similar to those reported by Gorinstein et al. (2005). Because 5% of Na_2SO_4 extracted less non-protein nitrogen than 29.3% NaCl, the former solution was chosen to extract proteins to prepare peptide fractions in this work.

3.2. DH and ACE-inhibitory activity of albumin 1 and globulin

Albumin 1 and globulin amaranth grain were hydrolysed with alcalase; progress of the hydrolysis was monitored by measuring the extent of proteolytic degradation through the DH according to the TNBS reaction with free amino groups (Fig. 2a and b). The albumin 1 and globulin DH increased sharply until 24 h (57 ± 0.019%) and 27 h (38 ± 0.06%), respectively. It was observed that prolonging the reaction time did not produce any significant improvement in the DH. Albumin 1 was easily hydrolysed by alcalase treatment, but globulin needed more time to be hydrolysed. Nevertheless, globulin appears to be somehow protected by the alcalase treatment. A similar behaviour was reported by Konishi et al. (1991): they observed that albumin 1 was decomposed by pronase, but not albumin 2 (actually globulin-*p*) because the latter is associated with protein-bodies. Profiles by SDS–PAGE have shown that the isolated protein-bodies from amaranth grain contain globulin and albumin 2. Perhaps other enzymes should be assayed in order to afford a faster globulin hydrolysis. According to Silva-Sánchez et al. (2008) globulins and glutelins are predicted to have in their primary sequences antihypertensive peptides, but not albumin 1.

Initially, albumin 1 and globulin fractions observed no ACE-inhibitory activity (Table 2). However, the hydrolysates of those protein fractions did show ACE-inhibitory activity (Fig. 2a and b). Once alcalase was added, the ACE-inhibitory activity increased gradually as alcalase hydrolysis proceeded. At the beginning of the hydrolysis, ACE inhibition increased with the DH up to a maximum inhibition at 18 h (44 ± 0.07% DH) and 15 h (13 ± 0.25% DH) for albumin 1 and globulin fractions, respectively. The data correspond to a 40 ± 0.03% and 35 ± 0.30% of ACE-inhibitory activity for albumin 1 and globulin, respectively. Under these conditions, the peptide fractions showed the highest IC_{50}, being 1.16 ± 0.05 mg/ml for albumin 1 and 0.63 ± 0.03 mg/ml as well as 0.69 ± 0.02 mg/ml for globulins G1 and G2, respectively. These values were calculated from their respective inhibition curves. In this step the enzymatic hydrolysates of both protein fractions showed
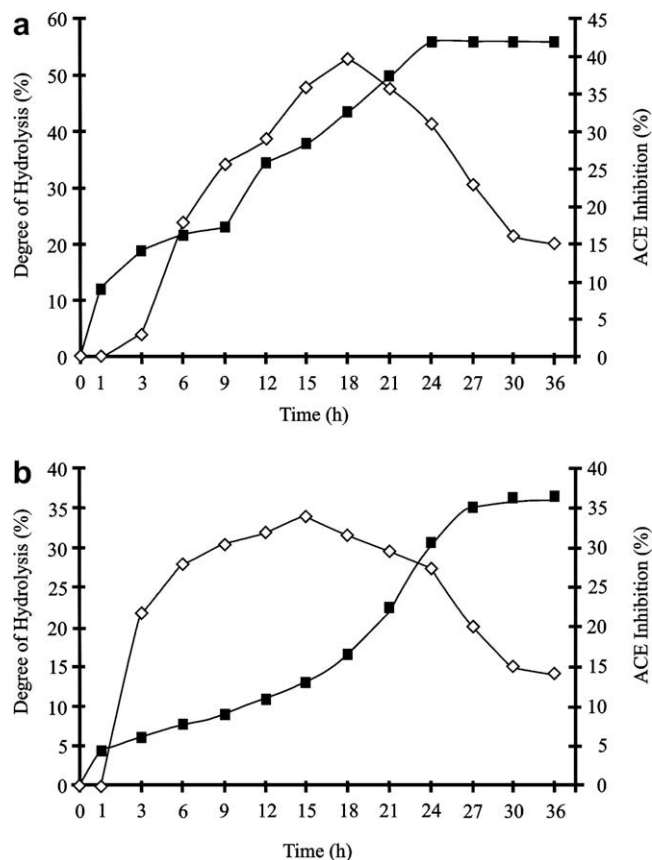
Fig. 2. DH (–■–) and % ACE-inhibitory activity (–◇–) as a function of time course of hydrolysis for albumin 1 (a) and globulin (b) with alcalase (0.8 AU/g protein).

Table 2
IC_{50} values of both albumin 1 and globulin collected fractions from gel filtration.

Fraction	IC_{50}[a] (mg/ml)
Albumin 1 (AI)	ND[b]
AI.1 digest	1.16 ± 0.05
AI.1.1	2.65 ± 0.12[c]
AI.1.2	0.82 ± 0.07
Globulin (G)	ND
G1	0.63 ± 0.3
G2	0.69 ± 0.02
G1.1	2.40 ± 0.02
G1.2	1.13 ± 0.06
G2.1	0.87 ± 0.09
G2.2	0.32 ± 0.02
Captopril	6.90 ± 0.32 ng/ml
ACE inhibitor	329 ± 26 ng/ml

[a] The IC_{50} value was determined by linear regression analysis of ACE inhibition (%) against Log (peptide concentration, mg/mL).
[b] ND = not determined, negligible ACE inhibition at the assayed conditions.
[c] Data are average ± standard deviation of triplicate determinations.

moderate ACE-inhibitory activity when compared with soy protein peptides ranging from 0.021 to 1.73 mg/ml. It is well known that most food-protein-derived antihypertensive peptides with high ACE-inhibitory activity must be purified and have a relatively low molecular weight (Mr; Philanto-Leppälä, Koskinen, Piilola, Tupasela, & Korhonen, 2000). Further hydrolysis of albumin 1 and globulin resulted in hydrolysates with decreased inhibition on ACE. Additionally, the hydrolysis time observed in this study for albumin 1 and globulin fractions was much longer than that used in most studies. Under the assayed experimental conditions, it took a very long time for albumin 1 and globulin to undergo alca-

lase hydrolysis when compared to other food proteins such as chickpea (30% DH and 40% ACE–IA at 30 min; Pedroche et al., 2002) and mung-bean (16% DH and 80% ACE-inhibitory activity at 2 h; Li et al., 2005).

Alcalase® is a serine bacterial endopeptidase (generic name subtilisin Carlsberg) prepared from a strain of *Bacillus licheniformis* with a specific activity of 2.4 AU/g (Anson units). Alcalase is a food grade enzyme which is also advantageous because of its lower cost, when it comes to its industrialised production, than that of pepsin, trypsin, chymotrypsin, papain or thermolysin, which have previously been used for the preparation of ACE-inhibitory peptides from food proteins. Subtilisin Carlsber hydrolyses peptide bonds with broad specificity, liberating peptides with hydrophobic amino acids such as Phe, Tyr, Trp, Leu, Ile, Val and Met at their C-terminal. Pedroche et al., 2002 have claimed that it is necessary to use more than one protease to obtain extensive hydrolysates with DH greater than 50%. In general, one enzyme cannot achieve such a high DH in a reasonable period of time. Pre-digestion with the endoprotease alcalase increases the number of N-terminal sites, facilitating hydrolysis by the exopeptidase flavourzyme. Nevertheless, further digestion of alcalase hydrolysis with flavourzyme did not show any marked effect on ACE inhibition, although it increased the DH. Alcalase has been used in the past for the production of ACE-inhibitory peptides (Matsufuji et al., 1994); thus this enzyme is the only one assayed in this work. These results indicated that there is an optimal degree of hydrolysis, above which more ACE-inhibitory peptides are degraded, but fewer ones are formed, thus decreasing the overall ACE-inhibitory activity. Moreover, ACE-inhibitory peptides produced by alcalase from sardine muscles are resistant to digestion by gastrointestinal proteases (Matsufuji et al., 1994) which would otherwise allow for absorption of peptides contained

in this sort of hydrolysates. Many of these hydrolysates have been shown to have antihypertensive activity when assayed in spontaneously hypertensive rats (SHR).

3.3. Purification of peptide fractions with ACE-inhibitory activity

Since 18 h albumin 1 and 15 h globulin hydrolysates afforded the maximum ACE inhibition, these fractions were selected for further purification and measurement of ACE-inhibitory activity. Fig. 3a and b show the gel filtration profile (Sephadex G-200) of albumin 1 and globulin fractions respectively, as well as the previously mentioned hydrolysates (18 and 15 h). Albumin 1 showed three main peaks: A1 (313 kDa), A2 (159 kDa) and A3 (101 kDa). These three albumin 1 fractions were analysed by their SDS–PAGE (data not shown) to determine their polypeptide profile. Since it was difficult to separate each peak individually from the gel filtration column, due to the close elution of A1, A2 and A3, the initial albumin 1 fraction (AI) was used. When the albumin hydrolysate (18 h) was analysed, it resulted in a broad peak (AI.1; <1.35 kDa). The AI.1 polypeptide fraction was subsequently applied to a Sephadex G-15 column to analyse its polypeptide composition (Fig. 3a'). Thus the fractionation of AI.1 resulted in the AI.1.1 (4.7 kDa) and AI.1.2 (0.55 kDa). If 120 g/mol is assumed to be the mean Mr for an amino acid, the likely peptide length would be calculated implying 4 to 5 amino acids for AI.1.2. Similarly, the globulin fraction was eluted from Sephadex G-200 obtaining two fractions. The result of fractionation was found to be the peaks of G1 (457 kDa) and G2 (41 kDa). Separately, a further fractionation of G1 and G2 after 15 h hydrolysis resulted in the G1.1 and G1.2 and, in turn, G2 afforded G2.1 and G2.2. According to their elution pattern from Sephadex G-15 (Fig. 3b'), G1.1 (7.5 kDa), G1.2 (4.7 kDa), G2.1
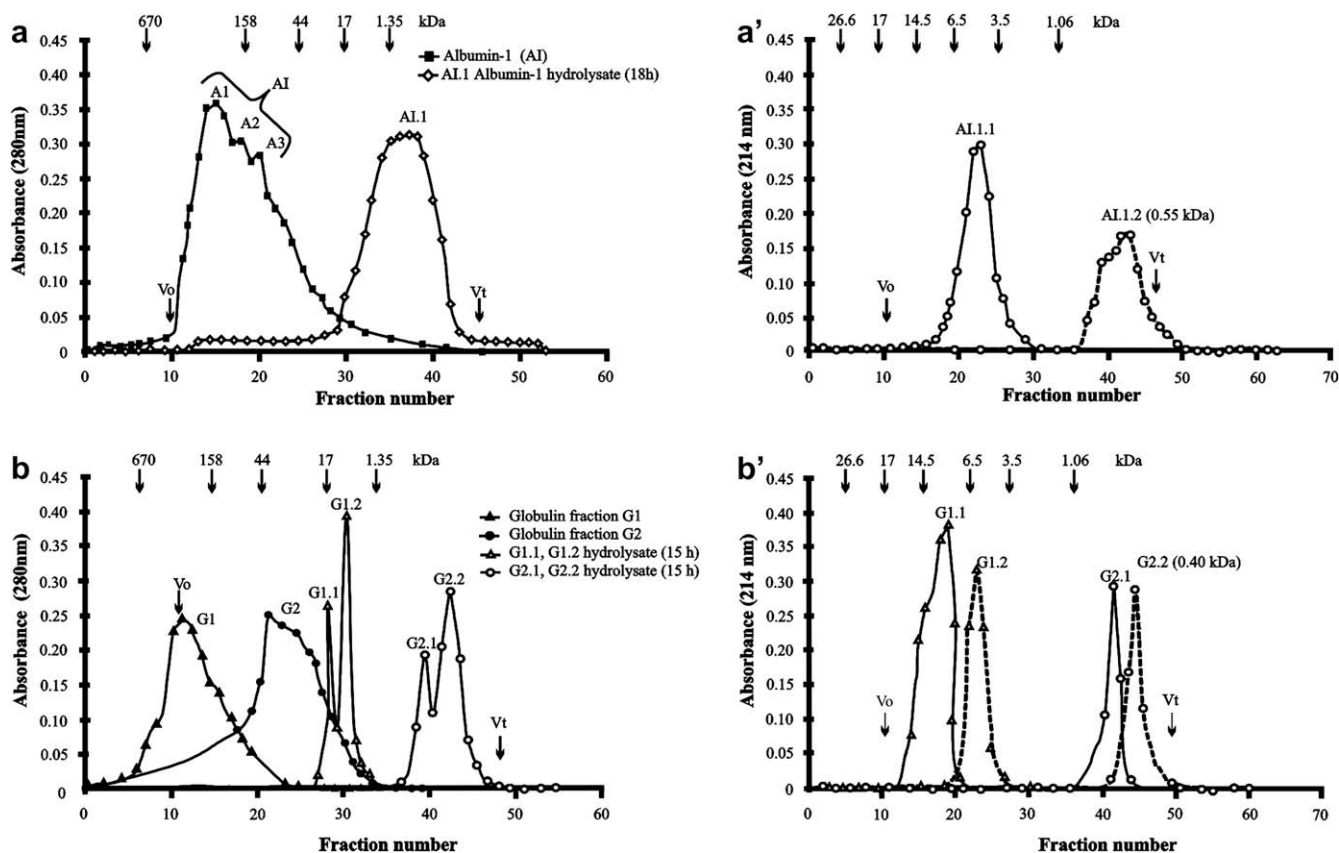

Fig. 3. Gel filtration profiles for albumin 1 and globulin as well as their respective hydrolysate fractions. (a) and (b) show the fractions when they were eluted from Sephadex G-200 column, (a') and (b') are the fractions eluted from Sephadex G-15 column. kDa, molecular weight of the standard proteins.

(0.55 kDa) and G2.2 (0.4 kDa). The smallest peptide fraction (G2.2) obtained may be composed of di- or tripeptides. It is well known that most food-protein-derived ACE-inhibitory peptides have relatively low Mr, which corresponds to the mass of 2–12 amino acid residues.

The fractions collected from gel filtration were tested for % inhibition against ACE. The IC_{50} values of albumin 1 and globulin peptides are shown on Table 2. The enzymatic hydrolysates of albumin 1 and globulin peptides had moderate ACE-inhibitory activity i.e. AI.1.2 (IC_{50} = 0.82 ± 0.07 mg/ml) and G2.2. (IC_{50} = 0.32 ± 0.02 mg/ml). Fractions AI.1.2 and G2.2 from the Sephadex G-15 column, which registered the highest IC_{50} value in its respective protein fraction, were applied to a RP–HPLC column to be separated on the basis of non-polarity. The elution profile is shown in Fig. 4a and b. Fractions AI.1.2 and G2.2 were eluted from the RP–HPLC column as four fractions and assayed for their IC_{50} values: fractions AI.1.2.1, AI.1.2.2, AI.1.2.3 and AI.1.2.4 eluted at 4.5, 9, 14 and 30% (v/v) acetonitrile, respectively. Similarly, fractions G2.2.1, G2.2.2, G2.2.3 and G2.2.4 eluted at 3, 8, 17 and 30% acetonitrile, respectively. Because of the negligible ACE-inhibitory activity% for AI.1.2.1; AI.1.2.3; AI.1.2.4; G2.2.1; G2.2.3 and G2.2.4, their IC_{50} value was not investigated. IC_{50} was 0.35 ± 0.02 mg/ml for the AI.1.2.2 peptide fraction, whereas the G2.2.2 peptide fraction was 0.15 ± 0.03 mg/ml. Considering a Mr of 550 Da for AI.1.2 and 400 Da for G2.2, as deduced from Sephadex G-15 chromatography, the IC_{50} values would correspond to 636 μM and 375 μM. The ACE-inhibitory activity of captopril and the ACE inhibitor (pGlu–Trp–Pro–Arg–Pro–Gln–Ile–Pro–Pro) were compared to that of AI.1.2 and G2.2 (Table 2). Captopril and the ACE inhibitor have a stronger ACE-inhibitory activity than that of amaranth peptides. The IC_{50} was 6.9 ± 0.32 ng/ml for captopril and 329 ± 26 ng/ml for the ACE inhibitor, which compares closely to the reported IC_{50} value of

1.3–8.9 ng/ml for captopril and 100–300 ng/ml for the ACE inhibitor (Lo & Li-Chan, 2005). Once the reports on enzymatic hydrolysates of various proteins were reviewed, the ACE-inhibitory activities were observed with the IC_{50} values ranging from 0.20 to 246.7 mg/ml (Li, Le, Shi, & Sherstha, 2004). Some tripeptides that are isolated from the hydrolysate of α-zein, a maize endosperm protein, act as ACE inhibitors. The IC_{50} values for these peptides (Leu–Arg–Pro, Leu–Ser–Pro, Leu–Gln–Pro and Leu–Ala–Tyr) were 0.27, 1.7, 1.9 and 3.9 μM, respectively. When a soy peptide fraction was purified, four peptides were found with IC_{50} values ranging from 39 to 153 μM. Particularly, the IC_{50} values of proline peptide at the C-terminal were around 1–82 μM. Therefore, the results of this study suggest that G2.2 globulin peptide fraction may have one of the most active naturally-occurring ACE-inhibitory peptides (IC_{50} = 0.32 ± 0.02 mg/ml or 800 μM). As far as a single purified peptide could be obtained, this value would increase. Therefore, the results also suggest that peptides with low Mr that are hydrophobic may have a stronger inhibition pattern against ACE. It should be noted that in this work G2.2.2 is a mixture of peptides, not a single purified peptide. Additionally, if a single peptide were isolated, its IC_{50} value might be raised.

3.4. Inhibitory kinetics of amaranth derived ACE-inhibitory peptide fractions

Heart diseases such as arteriosclerosis, coronary heart disease, stroke, peripheral arterial disease, and heart failure, may be caused by hypertension or blood pressure higher than 140 mm Hg systolic and/or 90 mm Hg diastolic pressure. ACE plays a key role in the rennin–angiotensin system, which regulates arterial blood pressure as well as salt and water balance. ACE inhibitors such as captopril, benazepril, enalapril, fosinopril, lisinopril, moexipril, preindopril, quinapril, ramipril, and trandolapril are the most commonly prescribed drugs (Sica, 2003). However, it is reported that captopril has several side effects such as hypotension, increased potassium levels, reduced renal function, cough, angioedema, skin rashes, taste disturbances as well as alterations in serum lipid metabolism; it may also cause fetal abnormalities. Since hypertensive patients need life-long medical treatment, there is an increasing interest in finding alternative ways to treat this condition that will avoid the undesirable side effects associated with ACE-inhibitory drugs. Thus, there is a strong need to identify natural inhibitors of ACE within the primary sequences of a range of food proteins. Because of the inhibitory kinetic properties of natural ACE, inhibitors have been the subject of research efforts to elucidate the mechanism of action of the peptides. The mode of inhibition of the ACE-catalysed hydrolysis of Hip–Leu–His was evaluated by a Lineweaver-Burk curve. The initial velocity was obtained after
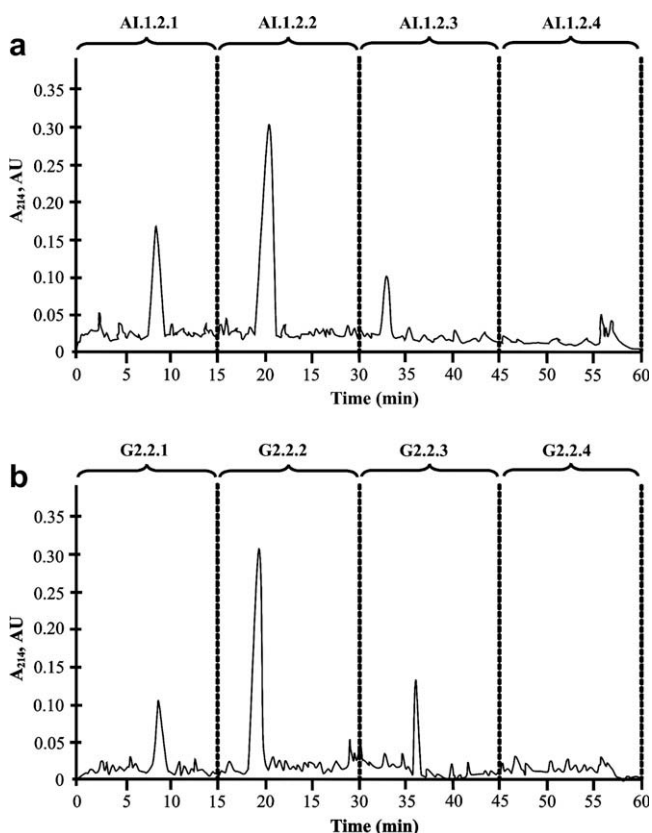

Fig. 4. RP–HPLC profile for: (a) fraction AI.1.2 and (b) fraction G2.2 from Sephadex G-15 column.
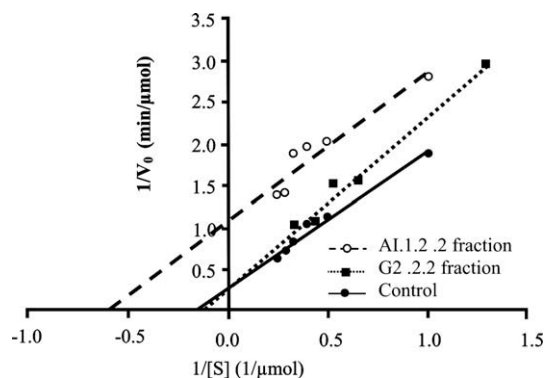

Fig. 5. Lineweaver-Burk plots of ACE inhibition for albumin 1 and globulin amaranth peptide fractions: (a) AI.1.2.2 and (b) G2.2.2, respectively.

plotting hippuric acid chromogen concentration against enzymatic reaction time. Albumin 1 derived peptides (AI.1.2.2) showed uncompetitive inhibition, but globulin derived peptides were competitive inhibitors for G2.2.2 (Fig. 5). Although uncompetitive ACE-inhibitory peptides have been reported (Matsufuji et al., 1994; Nakagomi et al., 2000), most ACE inhibitors from snake venom and derived from food-protein hydrolysates belong to the competitive mode. These competitive inhibitors are able to enter the ACE protein molecule, interact with the active sites and prevent substrate binding. In addition, a bioinformatic analysis performed by Silva-Sánchez et al. (2008) claimed that the location of the antihypertensive peptides is only found in the globulin sequence, that is, di- and tripeptides as Leu–Phe; His–Tyr; Gly–Lys–Pro and Arg–Phe. As shown above, albumin 1 peptides were also identified as having IC_{50} values similar to the globulin ones for the purposes of this study. They might be composed of 4 to 5 amino acids whereas globulin peptides might be composed of 3 amino acids. Due to the high absorption at 280 nm by means of RP–HPLC (data not shown), all the peptides from albumin 1 and globulin might be composed of hydrophobic and aromatic amino acids because the alcalase hydrolyses peptide bonds with broad specificity, thus liberating peptides such as Phe, Tyr, Trp, Leu, Ile, Val and Met at their C-terminal. The C-terminal residues of ACE-inhibitory peptides play a predominant role in competitive binding to the active site of ACE. It has been reported that peptides with hydrophobic and aromatic amino acids at the C-terminal are amongst the most favourable for strong competitive binding to ACE (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980). It was suggested that most active naturally-occurring ACE-inhibitory peptides contain proline or aromatic acid residues (Kawakami & Kayahara, 1993). There is currently a binding model for interactions between the substrate and active site of ACE. The C-terminal tripeptide residues may interact with the sub-sites S_1, S_1', and S_2' 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. Enzymes such as pepsin cleave at the carboxyl end of hydrophobic and aromatic amino acids (Phe, Tyr, Trp, and Leu); they also result in peptides with those amino acids at the C-terminal (Nelson & Cox, 2000). The fact that there are two active enzymatic sites in ACE may explain their wide substrates or inhibitor specificity (Perich, Jackson, Rogerson, Mendelsohn, & Johnston, 1992). These peptides bind tightly to ACE at its active site and compete with angiotensin I to convert it to angiotensin II (Cushman et al., 1987). Because of this, researchers have focused their interest on the isolation and identification of purified ACE-inhibitory peptides from various food sources after enzymatic digestion. Further studies using enzymes (Lo & Li-Chan, 2005) such as pepsin, papain, trypsin, pancreatin, *B. subtillis* protease, and pepsin, which have been used individually for the hydrolysis of soy, should be performed to discover amaranth peptides with hydrophobic and aromatic amino acids at the C-terminal. Nevertheless some food-protein-derived ACE inhibitors failed to show hypotensive activity after oral or intravenous administration *in vivo* due to the fact that they are substrates of ACE or hydrolysed in the gastrointestinal tract to peptides with reduced activity (Li, Le, Shi, & Sherstha, 2004); some peptides with weak ACE-inhibitory activities *in vitro* show strong antihypertensive activities after their consumption. Therefore, further research is needed to examine whether amaranth derived peptides may exert antihypertensive activity *in vivo*.

4. Conclusions

The study confirmed that bioactive peptide fractions can be released from amaranth grain proteins by alcalase-mediated digestion. Amaranth proteins are good raw material for production of ACE-inhibitory peptides. The peptide fractions showing ACE-inhib-

itory activity are a mixture of peptides: if they were purified, single peptides would be found with higher IC_{50} values. However, from the point of view of commercialisation, a crude protein hydrolysate should prove to be an economic way to consume these hypotensive peptides for life-long medical treatment. Further studies are needed on the characterisation of the amino acid composition and peptide sequence of the protein hydrolysates, the elucidation of the relationship between peptide structure and activity as well as the optimisation of enzymatic hydrolysis using several enzymes different to alcalase. Studies *in vivo* using SHR rats are also needed and, above all, their application in formulas of functional foods. So far, and to the best of our knowledge, this is the first effort to obtain albumin 1 and globulin peptides from amaranth grain with ACE-inhibitory activity using alcalase.

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