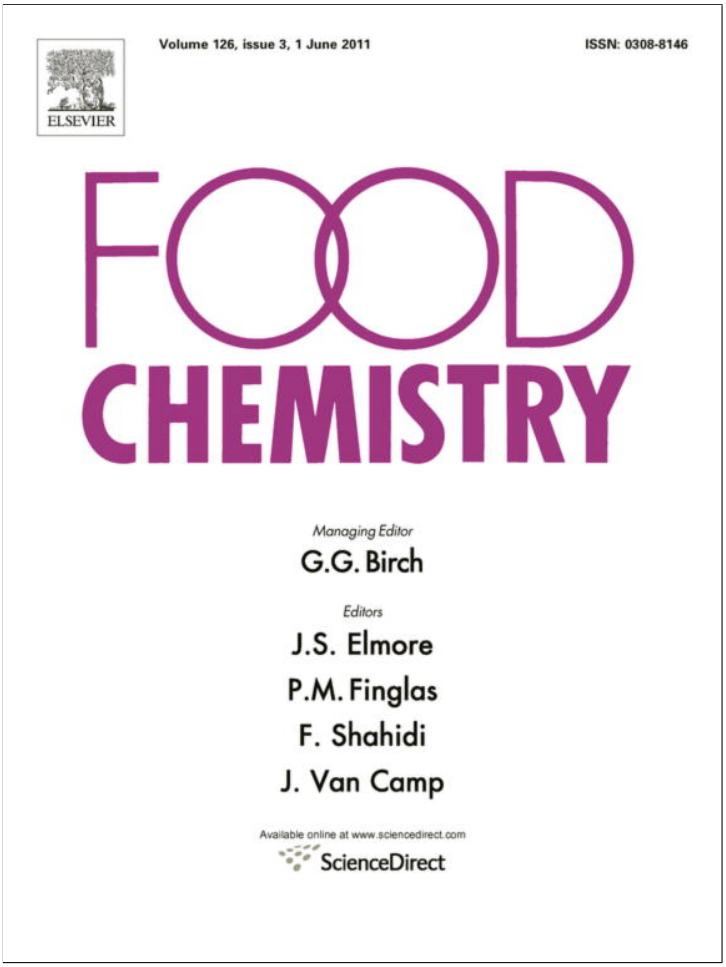

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Amaranth seed protein hydrolysates have in vivo and in vitro antihypertensive activity

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ABSTRACT

The objective of this work was to study the hydrolytic release of encrypted peptides with antihypertensive activity from storage proteins of *Amaranthus mantegazzianus*, as determined by in vitro assays, for the first time by in vivo studies in animal models, and by ex vivo assays. Hydrolysates with hydrolysis degree (DH) of 45% and 65% (IC50 0.12 mg/ml, equivalent to 300–600 μM) exhibited an angiotensin-I converting enzyme 1 (ACE) inhibitory activity equal or higher than the potential inhibitory of the average antihypertensive peptides registered in the BIOPEP database and of semi-purified *Amaranthus hypochondriacus* albumin and globulin protein fractions. Intragastric administration of hydrolysates with DH of 45% was effective in lowering blood pressure of male spontaneously hypertensive rats (SHR). Experiments performed in papillary muscles isolated from hearts and with isolated aortic smooth muscle of SHR suggest that the hypotensive effect could be attributed to a lowering of the peripheral resistance. We assume that the amaranth hydrolysates would be acting at the level of the local or autocrine renin–angiotensin system (RAS).

1. Introduction

Hypertension is defined as a sustained elevation of arterial blood pressure, which is associated with an increased risk of cardiovascular disease. It is one of the most frequent chronic conditions in developed countries, and its incidence is only increasing. An estimated 20% of the adult population suffers hypertension. The prevalence of elevated arterial pressure increases with age; in Western countries it affects around 65% of people aged 65–75 years (Alper, Calhoun, & Oparil, 2001). ACE (angiotensin-I converting enzyme 1), a dipeptidyl carboxypeptidase, is a key component of the renin–angiotensin system that regulates not only arterial pressure but also the water and electrolyte balance. ACE inhibition induces a decrease in arterial pressure by increasing the levels of the vasodilator bradykinin and reducing the levels of the vasoconstrictor angiotensin II (Rice, Thomas, Grant, Turner, & Hooper, 2004). A group of drugs, such as captopril and enalapril exploit this inhibitory approach for controlling hypertension (Dell'Itallia, Rocic, & Lucchesi, 2002).

Two of the most important tools for the effective treatment of the disease are diet modification and drug therapy. An increase in protein consumption seems to decrease arterial pressure in hypertensive patients. Although several mechanisms may operate, ACE inhibition by bioactive peptides may be partially responsible for this improvement in arterial pressure. In the past years, it has been shown that food proteins constitute a source of natural blood-lowering peptides with in vivo activity, milk proteins being the most thoroughly studied (Murray & FitzGerald, 2007).

In addition, some studies in hypertensive patients have demonstrated the antihypertensive effect of inhibitory peptides from diverse sources or from functional foods that contain such bioactive compounds (Mizuno, 2005; Seppo, Jauhiainen, Poussa, & Korpela, 2003; Tuomilehto et al., 2004). These studies have prompted the marketing of several formulations of nutraceutic foods, particularly fermented milks that can moderate hypertension and reduce cardiovascular risk, among other effects (Sibbel, 2007).

Amaranth is a pseudocereal of the Amaranteacea family endowed with several advantages regarding culture characteristics – fast grow and drought resistance – and nutritional properties – storage proteins from seeds possess an excellent amino acid balance (Gorinstein et al., 2002). Amaranth can be incorporated not only to the diet of normal individuals but also to that of coeliac patients and people allergic to cereals.

Silva-Sánchez et al. (2008) have shown theoretically the existence of different bioactive peptides, including blood-lowering ones, in several storage proteins of amaranth grains. More recently,

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Vecchi and Añón (2009) have shown by an in silico approach the interaction of ACE with two new tetrapeptides only found in amaranth 11S globulin, which can inhibit the activity of such enzyme in vitro. In addition, Tovar-Pérez, Guerrero-Legarreta, Farrés-González, and Soriano-Santos (2009) have recently shown the existence of ACE-inhibitory peptides in the albumin 1 and globulin fractions from *Amaranthus hypochondríacus*.

The main goal of the present work was to study the hydrolytic release of encrypted peptides from storage proteins present in amaranth protein isolates, with antihypertensive activity as determined by in vitro assays – ACE inhibition, for first time in vivo studies in animal models, and ex vivo assays. The potential mechanism of action of such peptides was also studied.

2. Materials and methods

2.1. Chemicals

All chemicals and solvents were of analytical grade and were obtained from either Merck & Co., Inc. (Whitehouse, NJ) or Sigma–Aldrich (St. Louis, MO).

2.2. Starting material

Seeds from *Amaranthus mantegazzianus* (cv. Pass, Don Juan variety) were grown and harvested in the Agronomy School of the National University of La Pampa, Argentina. Flour derived from these seeds was prepared in the Cereal Culture Service of the School of Agronomy and Forestry Sciences at the University of La Plata, Argentina. For that purpose, whole seeds were milled in a Udy mill with 1 mm mesh and passed through a screen of 10xx mesh. To eliminate fat, the flour was suspended at 10% w/v in *n*-hexane and was subjected to continuous stirring at 4 °C for 24 h. The flour was separated from the *n*-hexane by filtration and subsequent drying at room temperature. The defatted flour was stored at 4 °C until use.

2.3. Protein isolates preparation

Amaranth native isolates were prepared from defatted flour following the technique of Martínez and Añón (1996). The flour was suspended at 10% w/v in bidistilled water, and 2 M NaOH was added to reach pH 9. Proteins were extracted by incubating the suspension for 30 min at room temperature. The dispersion obtained was centrifuged for 20 min at 11,000*g* at 20 °C. The supernatant was harvested and proteins were precipitated by adding 2 M HCl until reaching pH 5. After centrifuging at 10,000*g* for 20 min at 4 °C, the sediment was separated and suspended in bidistilled water. The suspension was adjusted to neutral pH, freeze-dried, and stored at 4 °C until use.

The isolates were prepared at least in triplicate.

2.4. Hydrolysis reaction

The native isolate was suspended at 10% w/v in 75 mM phosphate buffer pH 7.8 with continuous shaking during 1 h at 37 °C in an orbital shaker with thermostat. Then, a protease solution was added and the mixture was incubated for varying times. The reaction was stopped by heating at 85 °C during 10 min; after this heating each sample was immediately frozen and freeze-dried. The proteases used were pronase (Pr), papain (P), trypsin (Tr), chymotrypsin (Ct) and alcalase (Alc) all of them acquired from Sigma–Aldrich (St. Louis, MO). The hydrolysis treatments were performed, at a minimum, in triplicate.

2.5. Determination of degree of hydrolysis

The concentration of free amino groups was measured with the trinitrobenzenesulfonic acid (TNBS) method (Adler-Nissen, 1979) in order to determine the degree of hydrolysis attained.

From the degree of hydrolysis, the average peptide length was estimated using:

$$L = \frac{100}{\mathrm{DH}}$$

where L is the average peptide length and DH is the degree of hydrolysis. This approximation holds for DH values higher than 10% (Adler-Nissen, 1979). The average molecular weight of the peptides in the hydrolysates could be further estimated with:

$$M = L \cdot M_{\mathrm{aa}}$$

where M is the molecular weight of the hydrolysate, L is the average length of the peptides, and M_{aa} is the average molecular weight of the aminoacids, weighed by their abundance in the amaranth protein isolate and equal to 130.

2.6. In vitro system

2.6.1. ACE assay

Angiotensin converting enzyme inhibition assay was performed using the method of Cushman and Cheung (1971) as modified by Kim, Yoon, Yu, Lönnerdal, and Cheung (1999). Briefly, 20 μl of peptide sample was added to 0.1 M potassium phosphate containing 0.3 M NaCl and 5 mM Hippuryl Histidyl Leucine (HHL), pH 8.3. ACE (5 mU) was added and the reaction mixture was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 0.1 ml 1 M HCl. The hippuric acid formed was extracted with ethyl acetate, heat evaporated at 95 °C for 10 min, dissolved in distilled water, and measured spectrophotometrically at 228 nm. ACE was acquired from Sigma–Aldrich (St. Louis, MO); HHL from MP Biomedicals (Santa Ana, CA,).

The inhibition percentage was calculated as

$$\%\mathrm{Inh} = 100[1 - (A_{\mathrm{m}} - A_{0\mathrm{m}}/A_{\mathrm{MAX}} - A_{0\mathrm{MAX}})]$$

where A_{m} and $A_{0\mathrm{m}}$ are the absorbance values of tubes with sample in the presence and absence of ACE, respectively. A_{MAX} and $A_{0\mathrm{MAX}}$ are the absorbance values in the absence of sample and the absence of both sample and ACE, respectively.

2.6.2. Determinations were made in triplicate

The IC50 (Inhibitory Concentration 50) was determined assessing the ACE inhibition of serial dilutions of each hydrolysate sample, and interpolating the protein concentration at which the inhibition percentage reached 50%.

2.7. In vivo system

2.7.1. Animals

A total of 52 male spontaneously hypertensive (SHR) rats and 10 normotensive Wistar rats, aged 3–4 months and weighing 220–270 g, were used in the course of this study. The animals were bred and maintained in the central facility of the National University of La Plata School of Medicine, and all the experimental procedures were supervised and approved by the Ethics Committee of the Institution. The rats were housed in stainless steel cages with sterilised bedding (white pine wood shavings) which was changed every day. They had air conditioning and a 12 h light–12 h dark cycle, and free access to food and water. Tap water was provided in sterilised bottles with stainless steel nipples. Food was in the form of extruded chips (Purina nutrients) of the following composition (in%): humidity 10, proteins 24.6, lipids 7, ashes 6.4, raw fibre 4,

hemicellulose 7.7, calcium 1.1, phosphorus 1.4, potassium 1.05, magnesium 0.2, sulphur 0.25, sodium 0.11, chloride 0.25, linoleic acid 2.25, linolenic acid 0.15, arginine 1.55, cysteine 0.42, glycine 1.4, histidine 0.59, isoleucine 0.97, leucine 2.25, lysine 1.2, methionine 0.4, phenylalanine 1.18, serine 0.09, threonine 0.9, valine 1.2, FDN 12, FDA 4.5, CNF 36, TDN 82.

2.7.2. Experiments with oral administration of hydrolysate and indirect measurement of blood pressure

Rats were placed in a chamber at 37 °C for 10 min, and then transferred to a standard setup (2008; Fritz & Rinaldi, 2007) with heating pad and acrylic restrainer, tail cuff and pulse sensor (Narco Biosystems, Houston, TX). The tail cuff was connected to a cylinder of compressed air through an arrangement of inlet and outlet valves that permitted inflation and deflation of the cuff at a constant rate. The tail cuff pressure was continuously recorded with a solid state pressure sensor (Sensym, All Sensors, Inc., San Jose, CA, USA). The signals from the pulse and pressure sensors were conveniently amplified and then digitised with an analogue–digital board (DT16EZ, Data Translation, Inc., Marlboro, MA) mounted in a desktop computer. On-line display for controlling the procedure, and files for later processing, were obtained with appropriate software (Labtech Notebook Pro, Laboratory Technology Corp., Wilmington, MA). The animals quickly became familiar with the procedure and remained calm within the restrainer, making unnecessary the use of the plastic door. In the rare cases when signs of discomfort were present the procedure was interrupted. After three control measurements, the amaranth hydrolysate was administered through small orogastric tubing with the aid of a mouth opener, and blood pressure was recorded hourly for the following 7 h.

2.7.3. Experiments with intragastric administration of hydrolysate and direct measurement of blood pressure

For direct blood pressure measurements, the rats were anesthetised with sodium pentobarbital (50 mg/kg I.P.) and placed on a heating pad. The left carotid artery was exposed and cannulated with a short catheter made of Microbore Tygon tubing (Cole-Parmer Scientific Co., Chicago) with a bonded Teflon end (OD 0.7 mm, Small Parts Inc., Miami Lakes, FL), following the technique described by Chen, Chandler, and Dicarlo (1995).

To administer the hydrolysate a small gastrostomy was performed through a tiny abdominal incision and a sylastic catheter 1.5 mm OD was advanced into the gastric lumen and fixed with a purse-string suture. Both the arterial and gastric catheters were exteriorised through the back of the neck, and the animals were fitted with rodent jackets, flexible springs and fluid swivels (Alice King Chatham Medical Arts, Hawthorne, CA) to allow free movement within the cage. All the studies were started 24 h after the surgical intervention, with the animals fully recovered. Arterial pressure was measured by connecting the carotid catheter to a pressure transducer (P23 Gb, Gould Inc., Cleveland, OH), and the amaranth hydrolysate was injected through the gastric tubing without causing any disturbance to the animal. Due to the damping of pressure waves caused by the catheters, only the mean blood pressure (MBP) is reported, and it was measured for up to 7 h in most animals.

2.8. Ex vivo assays

2.8.1. Experiments with isolated papillary muscle

Under anesthesia with sodium pentobarbital the thorax of rats was widely opened and the heart was rapidly excised and placed in cold physiological salt solution (PSS) of the following composition (in mM): NaCl 130, KCl 4.7, Na_2HPO_4 1.17, $MgSO_4$ 1.16, $NaHCO_3$ 24.0, $CaCl_2$ 1.6 and glucose 11.0, bubbled with a mixture of 5% CO_2 and 95% O_2. The left ventricular wall was opened along its longitudinal axis and a suitable papillary muscle was selected and isolated. One end was traversed with a small stainless steel pin, and the opposite was tied with a traumatic 6-0 silk to form a ring. The muscle was then excised and placed into a thermostatised chamber suffused with PSS at 37 °C (1 ml/min), and both ends were fixed to a stationary stainless steel hook and a miniature force transducer (Sensor One, Sausalito, CA, USA). The muscles were stimulated with depolarising pulses at 0.5 Hz through field electrodes, and were stabilised during 30 min. After that period the perfusion was continued for 60 min with a solution made of 0.25 g of amaranth hydrolysate in 250 ml of PSS. In each experiment a comparison was made between the developed force (F) and its first derivative ($\delta F/\delta t$) at the end of the stabilisation period and at the end of the hydrolysate perfusion.

2.8.2. Experiments with isolated aortic smooth muscle

After the heart was obtained from the rats described in the preceding paragraph, the thoracic aorta was excised a placed in cold PSS. The adjacent connective tissue was cleaned off, and aortic rings 2 mm long were cut. Special care was taken in not damaging the endothelial layer or over distending the vessel during this procedure. The rings were gently suspended between two stainless steel wires in water-jacketed organ baths kept at 37 °C and filled with PSS bubbled with a mixture of 5% CO_2 and 95% O_2, giving a pH of 7.40. The lower wire was fixed to a vertical plastic rod immersed in the organ bath, while the upper one was rigidly connected to a force transducer (Grass FT.03D, Grass Telefactor, West Warwick, CT, USA). The preparations were then stretched to obtain a passive force of ~2 g and stabilised during 1 h, changing the solution in the chamber every 20 min. At the end of that period the rings were exposed to increasing concentrations of norepinephrine (10^{-8} – 10^{-4} M) and the contractile responses in each step were recorded. In the control preparations, a washout was performed to regain the baseline, and a second concentration–response curve in PSS was performed. In the experimental preparations (obtained from the same rat), the second concentration–response curve was performed after incubation with a solution made of 0.25 g of amaranth hydrolysate in 250 ml of PSS. The responses in both situations were compared. In seven additional experiments, the concentration–response curves to norepinephrine were obtained after previous incubation with hydrolysate plus 100 mM of L-NAME to inhibit the endothelial production of NO.

2.9. Statistical analysis

The data obtained were statistically evaluated by variance analysis (ANOVA) if more than two groups were compared, or by t test if only two groups were compared. The comparison of means was done by the least significant difference (LSD) test at a significance level of 0.05 or 0.01. Both were carried out using Sigma Stat software.

3. Results

3.1. Antihypertensive activity of amaranth hydrolysates: in vitro assays

3.1.1. In vitro ACE inhibitory activity of amaranth protein hydrolysates

The percentage inhibition of ACE obtained with hydrolysates of the *A. mantegazzianus* isolate prepared with different proteases is shown in Table 1. The inhibitory activity of the non-hydrolysed isolate is higher than 0% ($P < 0.01$). Therefore, it can be concluded that the protein isolate of *A. mantegazzianus* contains species soluble at pH 8.3 and at high ionic strength capable of inhibiting ACE activity,

Table 1
ACE inhibitory activity of amaranth protein hydrolysates with different proteases and varying conditions.

Hydrolysate	% ACE inhibition	*N*
Protein isolate unhydrolysed	10.8 ± 2.1	7
Pronase 0.1 mg/ml 4 h	23.9 ± 4.2**	6
Papain 0.3 mg/ml 4 h	44.0 ± 5.2*	4
Trypsin 0.3 mg/ml 4 h	16.3 ± 1.5*	7
Chymotrypsin 0.3 mg/ml 4 h	53.9	1
Chymotrypsin 0.1 mg/ml 4 h	0.2 ± 4.1*	2
Trypsin 0.3 mg/ml 4 h	37.3 ± 11.5**	2
Trypsin 0.2 mg/ml 4 h	34.0 ± 6.4**	7
Trypsin 0.1 mg/ml 4 h	43.2 ± 2.4*	4
Trypsin 0.05 mg/ml 4 h	3.8 ± 2.5*	3
Alcalase 0.8 mg/ml 4 h	74.5 ± 2.0*	2

Statistically significant differences with the non-hydrolysed protein isolate are shown with $*P < 0.01$ and $**P < 0.001$. The third column shows the number of independent hydrolysate preparations assessed.

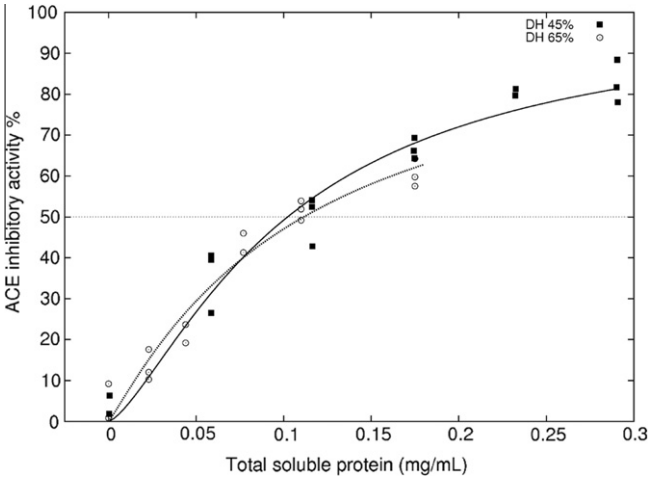

Fig. 1. Determination of the Inhibitory Concentration 50 (IC50) of alcalase hydrolysates of 45% (■) and 65% (○).

thus suggesting the presence of preexisting ACE inhibitory peptides. Tovar-Pérez et al. (2009) did not find an ACE inhibitory activity in the globulin and albumin I fraction of *Amaranthus hypochondriacus*, raising the possibility that the inhibitory activity detected in our protein isolate is located in a different fraction.

In most cases the proteolytic treatment was successful to produce hydrolysates with higher inhibitory activity. The exceptions were the chymotrypsin hydrolysates at 0.1 mg/ml for 4 h and the trypsin hydrolysates at 0.05 mg/ml for 4 h, which displayed a lower inhibitory activity than the non-hydrolysed isolate. The hydrolysate with the highest inhibitory capacity was the one obtained with alcalase 0.8 mg/ml 4 h. It is important to highlight that proteases from different origins – vegetal, animal, and microbial succeeded in releasing peptides with ACE inhibitory activity. This opens the possibility to the use of high-scale fermentation or enzymatic treatment techniques to produce functional foods based on amaranth protein isolates. It may also imply a potential local release of bioactive peptides in the gastrointestinal tract through protein digestion.

3.1.2. ACE inhibition vs. degree of hydrolysis

In order to obtain a hydrolysate with maximal ACE inhibitory activity in vitro, the effect of degree of hydrolysis on this biological activity was analysed. Alcalase was chosen based on the preliminary screening results shown above.

The ACE inhibitory capacity of alcalase hydrolysates with different degree of hydrolysis (DH) derived from *A. mantegazzianus* protein isolate was assayed. All the hydrolysates displayed an inhibitory activity substantially higher than that of the non-hydrolysed isolate; the isolate of 65% DH being the one with the highest activity. No statistically significant difference was found between the inhibitory potencies of hydrolysates of 17%, 28% and 45% DH ($P > 0.01$). These results confirm the effectiveness of the proteolytic treatment for the production of peptides with ACE inhibitory activity.

According to the results obtained by electrophoresis and size exclusion chromatography, the protein species present in the amaranth isolates that are degraded faster are the subunits of 7S globulin and 11S acidic subunits. The basic subunit of 11S is also degraded, but more gradually during the course of the reaction; it was still detected even at very high degrees of hydrolysis (65%). At such high degrees of hydrolysis there were still some species of molecular mass equal to o greater than 6 kDa although most of these peptides have MM under 10 amino acids (data not shown).

The dose–response curves for hydrolysates with 45% and 65% DH are shown in Fig. 1. The IC50 values obtained by interpolation of the data are indistinguishable, both being equal to 0.12 ± 0.02 mg/ml of soluble protein. Taking into account the average molecular weight of peptides in the hydrolysate, estimated from the DH value as described in Section 2, the IC50 values in molar base for hydrolysates with 45% and 54% DH were 415 μM and 600 μM, respectively. A comparison between these IC50 values and those of hydrolysates from other protein sources or purified ACE inhibitory peptides showed that values for amaranth hydrolysates are of the same order of magnitude than those usually found for food protein hydrolysates (0.08–1.08 mg protein/ml) (Hernández-Ledesma, Recio, Ramos, & Amigo 2002; Matsui, Li, & Osajima, 1999; Miguel, Recio, Gomez-Ruiz, Ramos, & Lopez-Fandino, 2004; van der Ven, 2002: Vecchi & Añón, 2009; Wanasundara et al., 2002; Wu, Aluko, & Nakai, 2006; Wu & Ding, 2002). Among the hydrolysates from vegetal sources, the ones examined in the present study have the lowest IC50. However, the maximal inhibitory potency corresponds to hydrolysates from milk proteins (IC50 0.081 mg/ml, Hernández-Ledesma et al., 2002). Compared with pure inhibitors, the IC50 values of amaranth hydrolysates are close to the mean of the IC50 distribution of the bioactive peptides database (BIOPEP). This means that there are purified peptides with higher inhibitory capacity than the hydrolysates here examined, but also several peptides with lower inhibitory potency. The hydrolysate is a heterogeneous mixture of several species, including polypeptides and peptides devoid of inhibitory activity and also amino acids, which may diminish the ACE inhibitory efficacy of the hydrolysate. Thus, it may be suggested that the hydrolysis has released ACE inhibitory peptides with IC50 values low enough to compensate such effect, resulting in a hydrolysate of moderate inhibitory potential.

3.1.3. Antihypertensive activity of amaranth hydrolysates: in vivo assays

In the assays performed in SHR with indirect measurement of blood pressure, the administration of either (1) vehicle only (water), (2) a tryptic hydrolysate of amaranth (2.5 g/kg of rat), or (3) a hydrolysate (2.4 g/kg of rat) obtained with alcalase (4 h incubation) failed to produce any significant effect on the blood pressure (Fig. 2). Only the injection of a hydrolysate (2.4 g/kg of rat) made with alcalase (5 h incubation) produced a significant decrease of blood pressure that was detected 3 h after the hydrolysate administration and persisted for 7 h. In the normotensive WKY rats none of the above mentioned hydrolysates showed any significant effect on blood pressure (data not shown).

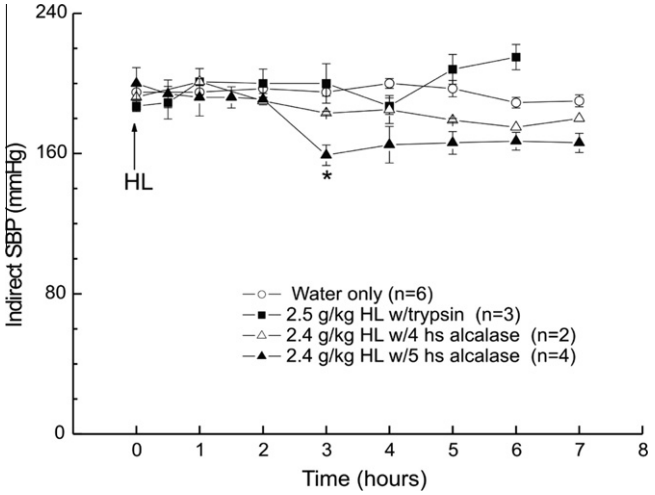

Fig. 2. Effect of various types of amaranth hydrolysate (HL) on systolic blood pressure (SBP) in conscious SHR (n = 15). In these experiments the hydrolysate was administered by orogastric intubation and the SBP was measured indirectly in the tail. Significant depression of SBP was obtained only with HL prepared with alcalase during 5 h.

In the assays performed in SHR with direct measurement of mean blood pressure the direct intragastric injection of 0.5 g/kg of amaranth hydrolysate produced moderate but non significant effects, and the blood pressure decreased further in a dose-dependent fashion as the hydrolysate was increased to 1.0, 1.5 and 2.4 g/kg body weight, reaching significance ($P < 0.05$) with the 1.0 and 1.5 g/kg concentrations (Fig. 3). The hypotensive effect was maximal 1.5 h after administration of the hydrolysate, and persisted for up to 7 h (Fig. 3). It must be noted, however, that the 7 h point for 2.4 g/kg includes two rats with BP measured indirectly. In several animals the catheter was patent for up to 5 days after the injection, and in these cases we were able to verify that the blood pressure had returned to control values by this time.

Fig. 4 shows the effect of 1 mg/ml of amaranth hydrolysate on isolated papillary muscles contracted by electrical stimulation. It can be seen that the exposure to the hydrolysate slightly depressed the contractile force, although the change did not reach significance ($P > 0.05$). The ($\delta F/\delta t$) was not changed by exposure to the hydrolysate (data not shown).

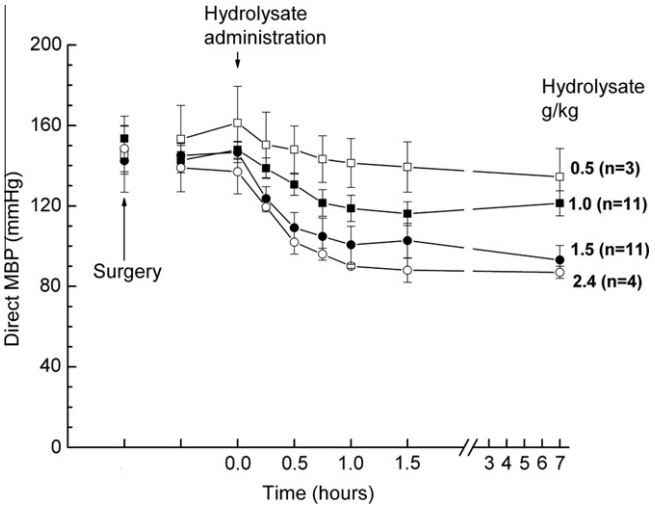

Fig. 3. Effect of amaranth hydrolysate prepared with alcalase during 5 h on mean blood pressure (MBP) in conscious SHR (n = 29). In these experiments the hydrolysate was administered by gastrostomy and the MBP was measured by carotid cannulation. Significant depression of MBP was obtained with hydrolysate in doses of 1.0, 1.5 and 2.4 g/kg.

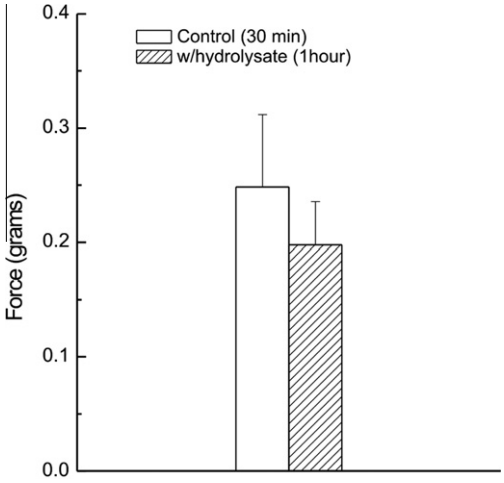

Fig. 4. Effect of amaranth hydrolysate prepared with alcalase during 5 h on the contractile force developed by left ventricular papillary muscles of SHR (n = 10), in control conditions (left bar) and after 1 h of exposure to hydrolysate. It can be seen that the force was slightly depressed but without reaching statistical significance.

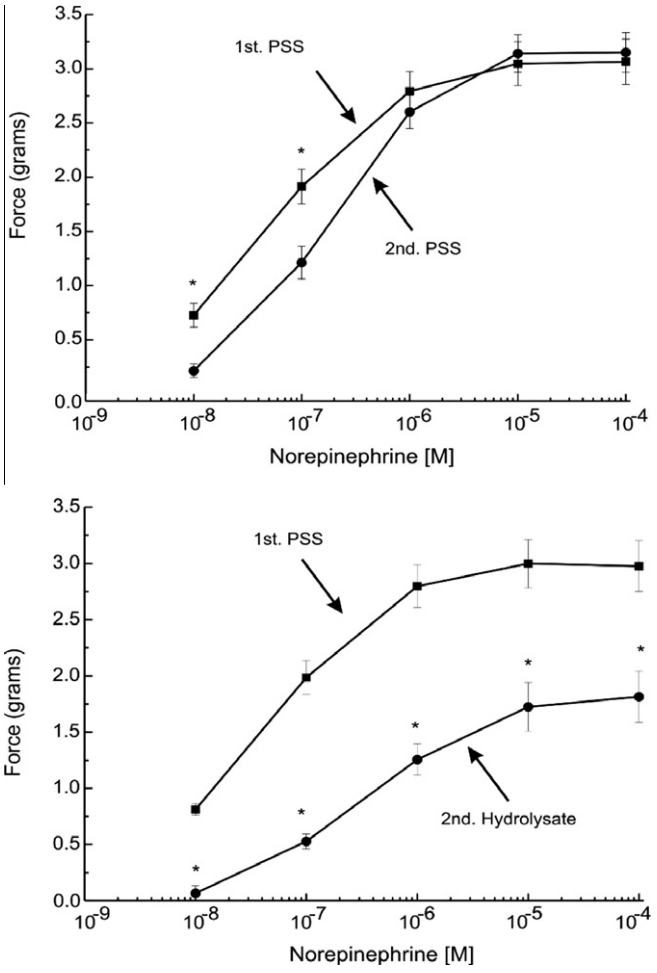

Fig. 5. Effect of amaranth hydrolysate on isolated aortic rings (n = 10) contracted by exposure to norepinephrine 10^{-8}–10^{-4} M. It can be seen that the repetition of the concentration-contraction curve in itself only caused a slight depression of the force at 10^{-8} and 10^{-7} M (top panel), but the exposure to the hydrolysate caused a significant depression of the contractile response at all the norepinephrine concentrations essayed (bottom panel).

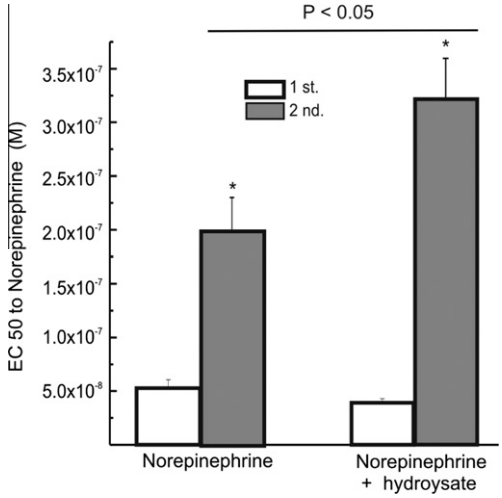

Fig. 6. IC50 to norepinephrine in isolated aortic rings (n = 10) contracted by exposure to norepinephrine 10^{-8}–10^{-4} M. The IC50 was significantly increased by the mere repetition of the concentration-contraction curve (left bars), but the exposure to the hydrolysate (right bars) induced an even greater increase of the IC50, which was significant with respect to the first.

Fig. 5 shows the effect of the same concentration of amaranth hydrolysate on isolated aortic rings contracted by exposure to norepinephrine 10^{-8} – 10^{-4} M. It can be seen that the repetition of the concentration–contraction curve during control experiments only caused a slight depression of the force at 10^{-8} – 10^{-7} M (top panel), but the exposure to the hydrolysate caused a significant depression of the contractile response at all the norepinephrine concentrations assayed (bottom panel).

Fig. 6 shows that the IC50 to norepinephrine was significantly increased by the mere repetition of the concentration–contraction curve, but the exposure to the hydrolysate induced an even greater increase, which was significant with respect to the former experiments ($P > 0.05$). In seven additional experiments, previous incubation of the rings with L-NAME 100 mM did not alter the results (data not shown).

4. Discussion

The results obtained during the present work show the existence in amaranth proteins of encrypted peptides endowed with antihypertensive activity, as was revealed by in vitro and for the first time by in vivo assays.

While the native isolate of *A. mategazzianus* has a measurable inhibitory activity in vitro, its enzymatic hydrolysis at DH > 15% produced a significant increase of such activity. This suggests the release of specific peptides endowed with ACE inhibitory activity as measured with the synthetic peptide used for in vitro assays in the present study. Although the hydrolysate with the highest inhibitory activity was the one with the highest DH, most of the increment in the inhibitory capacity, as compared to non-hydrolysed isolate, was obtained at moderate HDs. Hydrolysates with DH of 45% and 65% (IC50 0.12 mg/ml, equivalent to 300–600 μM) exhibited an ACE inhibitory activity equal or higher than that corresponding to semi-purified fractions from albumin a and globulin of *A. hypochondriacus* (Tovar-Pérez et al., 2009), and the potential inhibitory of the average antihypertensive, judging by the distribution of IC50 of the public database of bioactive peptides BIOPEP. These hydrolysates consisted mostly of peptides of less than five amino acids remaining some species of MM lower than 6 kDa. One or more of the peptides present in these hydrolysates, suitable for use as food ingredients, they must possess antihypertensive activity, cannot rule out the existence of synergistic or antagonistic effects between them. Ongoing studies in our laboratory and complement previous studies (Vecchi & Añón, 2009) are being made for the purposes of determining the identity of the main bioactive peptides.

The intragastric administration of amaranth hydrolysates (DH 45%) was also effective in lowering blood pressure of SHR in on the acute effects of such treatment, reaching its maximal effect in approximately 90 min. There was a difference between the effects obtained with oral administration and those produced by direct intragastric administration of the hydrolysate since 2.4 g/kg, the only oral dose that reduced blood pressure was twice as effective when injected directly to the stomach. Most probably this was due to the different procedures involved: the administration through a gastrostomy does not produce any discomfort in the rat, while the oral introduction of a plastic tubing, even if carefully performed, induces a considerable degree of stress. In addition, the hourly measurement of blood pressure involves first a preheating and then the immobilisation in a plastic chamber, and both maneuvers are prone to produce some degree of excitation. On the contrary, the direct measurement can be performed with the animal being totally unaware of the procedure.

Since the blood pressure is the product of cardiac output and peripheral resistance, its fall could have been due to the action of the hydrolysate on either of the two variables. In order to assess the possibility of a depressant effect of the hydrolysate on the myocardium, experiments were performed in papillary muscles isolated from hearts of the same species of rats. In these experiments we were not able to detect any significant negative inotropic effect, and in consequence it can be assumed that under "in vivo" conditions the heart is not affected and the decrease of peripheral resistance would be the cause of the fall in blood pressure.

Further experimental demonstration of this assertion is provided by the experiments in isolated aortic smooth muscle, in which the depression of the smooth muscle contractility was far greater than the effect on the myocardium. Even considering the fact that the aorta is not a resistance vessel the effect on the arterioles is likely to be similar, and thus the hypotensive effect could be attributed to a lowering of the peripheral resistance.

In our "in vitro" experiments we have demonstrated an important effect of amaranth hydrolysates as inhibitors of the angiotensin-converting enzyme, ACE, and this action can also explain our results in the SHR experiments. In the conscious animals we could detect a significant lowering effect on blood pressure that we suspect is primarily due to peripheral vasodilatation as outlined above. In this case we can assume that the peptides with ACE inhibitory activity are absorbed and exert their action at the level of the classical or "humoral" renin–angiotensin system (RAS). However, we were also able to detect a depression in the responses of papillary muscle (although not significant) and a relaxant effect on isolated aortic rings, which was endothelium-independent. Since these are isolated tissues, we must assume that the amaranth hydrolysates would be acting at the level of the local or autocrine RAS, which has been identified in various organs (Paul, Mehr, & Kreutz, 2006; Ruzicka & Leenen, 1997; Urata, Nishimura, & Ganten, 1996) and especially in the peripheral vascular system (De Mello & Danser, 2000; Dzau, 1988; Hirsch, Pinto, Schunkert, & Dzau, 1990). In addition, an intracellular chymase has been identified in the local RAS, with a role similar to that of the ACE, i.e. the conversion of angiotensin I in angiotensin II (Paul et al., 2006; Urata et al., 1996). It remains to be determined if the effect of the amaranth hydrolysates is exerted only on the ACE, as we demonstrated in our experiments, or if a chymase-inhibiting action is also present.

An important issue to be addressed in future studies is whether the hypotensive action here demonstrated "in vivo" with the intragastric administration of amaranth hydrolysates can be repro-

duced after the dietary administration of the cereal to the same animals. If further studies do confirm a hypotensive effect of the amaranth administered with the diet, it would open the possibility of its administration to human populations with therapeutic and/or preventive purposes.

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References

Adler-Nissen, J. (1979). Determination of degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of Agricultural and Food Chemistry, 27*, 1256–1262.

Alper, A. B., Calhoun, D. A., & Oparil, S. (2001). Hypertension. *In Encyclopedia of life sciences* (pp. 1–8). Nature Publishing Group. Available from: http://www.els.net.

Chen, Y. F., Chandler, M. P., & Dicarlo, S. E. (1995). Acute exercise attenuates cardiac autonomic regulation in hypertensive rats. *Hypertension, 26*, 676–683.

Cushman, D. W., & Cheung, H. S. (1971). Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochemical Pharmacology, 20*, 1637–1648.

De Mello, W. C., & Danser, A. H. J. (2000). Angiotensin II and the heart. On the intracrine renin–angiotensin system. *Hypertension, 35*, 1183–1188.

Dell'Itallia, L. J., Rocic, P., & Lucchesi, P. A. (2002). Use of angiotensin-converting enzyme inhibitors in patients with diabetes and coronary artery disease. *Current Problems in Cardiology, 27*(1), 6–36.

Dzau, V. J. (1988). Molecular and physiological aspects of tissue renin–angiotensin system: Emphasis on cardiovascular control. *Journal of Hypertension, 6*(Suppl. l), S7–12.

Fritz, M., & Rinaldi, G. (2007). Influence of nitric oxide-mediated vasodilation on the blood pressure measured with the tail-cuff method in the rat. *Journal of Biomedical Science, 14*, 757–765.

Fritz, M., & Rinaldi, G. (2008). Blood pressure measurement with the tail-cuff method in Wistar and spontaneously hypertensive rats: Influence of adrenergic- and nitric oxide-mediated vasomotion. *Journal of Pharmacology and Toxicology Methods, 58*, 215–221.

Gorinstein, S., Pawelzik, E., Delgado-Licon, E., Haruenkit, R., Weisz, M., & Trakhtenberg, S. (2002). Characterisation of pseudocereal and cereal proteins by protein and amino acid analyses. *Journal of the Science of Food and Agriculture, 82*, 886–891.

Hernández-Ledesma, B., Recio, I., Ramos, M., & Amigo, L. (2002). Preparation of ovine and caprine beta-lactoglobulin hydrolysates with ACE-inhibitory activity. Identification of active peptides from caprine beta-lactoglobulin hydrolysed with thermolysin. *International of Dairy Journal, 12*, 805–812.

Hirsch, A. T., Pinto, Y. M., Schunkert, H., & Dzau, V. J. (1990). Potential role of the tissue renin–angiotensin system in the pathophysiology of congestive heart failure. *American Journal of Cardiology, 66*, 22D–30D.

Kim, Y., Yoon, S., Yu, D., Lönnerdal, B., & Cheung, B. (1999). Novel angiotensin-I-converting enzyme inhibitory peptides derived from recombinant human αs1-casein expressed in *Escherichia coli*. *Journal of Dairy Research, 66*, 431–439.

Martínez, N. E., & Añón, M. C. (1996). Composition and structural characterization of amaranth protein isolates. An electrophoretic and calorimetric study. *Journal of Agricultural and Food Chemistry, 44*, 2523–2530.

Matsui, T., Li, C. H., & Osajima, Y. (1999). Preparation and characterization of novel bioactive peptides responsible for angiotensin I-converting enzyme inhibition from wheat germ. *Journal of Peptide Science, 5*, 289–297.

Miguel, M., Recio, I., Gomez-Ruiz, J. A., Ramos, M., & Lopez-Fandino, R. (2004). Angiotensin I-converting enzyme inhibitory activity of peptides derived from egg white proteins by enzymatic hydrolysis. *Journal of Food Protection, 67*(9), 1914–1920.

Mizuno, S. (2005). Antihypertensive effect of casein hydrolyzate in a placebo-controlled study in subjects with high-normal blood pressure and mild hypertension. *British Journal of Nutrition, 94*, 84–91.

Murray, B. A., & FitzGerald, R. J. (2007). Angiotensin converting enzyme inhibitory peptides derived from food proteins: Biochemistry, bioactivity and production. *Current Pharmaceutical Design, 13*, 773–791.

Paul, M., Mehr, A. P., & Kreutz, R. (2006). Physiology of local renin–angiotensin systems. *Physiology Review, 86*, 747–803.

Rice, G. I., Thomas, D. A., Grant, P. J., Turner, A. J., & Hooper, N. M. (2004). Evaluation of angiotensin-converting enzyme (ACE), its homologue ACE2 and neprilysin in angiotensin peptide metabolism. *Biochemical Journal, 383*, 45–51.

Ruzicka, M., & Leenen, F. H. H. (1997). Update on local cardiac renin–angiotensin system. *Current Opinion in Cardiology, 12*, 347–353.

Seppo, L., Jauhiainen, T., Poussa, T., & Korpela, R. (2003). A fermented milk high in bioactive peptides has a blood pressure-lowering effect in hypertensive subjects. *American Journal of Clinical Nutrition, 77*, 326–330.

Sibbel, A. (2007). The sustainability of functional foods. *Social Science and Medicine, 64*(3), 554–561.

Silva-Sánchez, C., Barba de la Rosa, A. P., León-Galván, M. F., de Lumen, B. O., de León-Rodríguez, A., & González de Mejía, E. (2008). Bioactive peptides in amaranth (*Amaranthus hypochondriacus*) seed. *Journal of Agricultural and Food Chemistry, 56*, 1233–1240.

Tovar-Pérez, E. G., Guerrero-Legarreta, I., Farrés-González, A., & Soriano-Santos, J. (2009). Angiotensin I-converting enzyme-inhibitory peptide fractions from albumin 1 and globulin as obtained of amaranth grain. *Food Chemistry, 15*, 437–444.

Tuomilehto, J., Lindström, J., Hyyrynen, J., Korpela, R., Karhunen, M., Mikkola, L., et al. (2004). Effect of ingesting sour milk fermented using *Lactobacillus helveticus* bacteria producing tripeptides on blood pressure in subjects with mild hypertension. *Journal of Human Hypertension, 18*, 795–802.

Urata, H., Nishimura, H., & Ganten, D. (1996). Chymase-dependent angiotensin II forming systems in humans. *American Journal of Hypertension, 9*, 277–284.

van der Ven, C. (2002). *Biochemical and functional characterisation of casein and whey protein hydrolysates*. PhD Thesis. Wageningen Universiteit, Netherlands.

Vecchi, B., & Añón, M. C. (2009). ACE inhibitory tetrapeptides from *Amaranthus hypochondriacus* 11S globulin. *Phytochemistry, 70*, 864–870.

Wanasundara, P. K., Ross, A. R., Amarowicz, R., Ambrose, S. J., Pegg, R. B., & Shand, P. J. (2002). Peptides with angiotensin I-converting enzyme (ACE) inhibitory activity from defibrinated, hydrolyzed bovine plasma. *Journal of Agricultural and Food Chemistry, 50*, 981–988.

Wu, J., Aluko, R. E., & Nakai, S. (2006). Structural requirements of angiotensin I-converting enzyme inhibitory peptides: Quantitative structure–activity relationship study of di- and tripeptides. *Journal of Agricultural and Food Chemistry, 54*, 732–738.

Wu, J., & Ding, X. (2002). Characterization of inhibition and stability of soy-protein-derived angiotensin I-converting enzyme inhibitory peptides. *Food Research International, 35*, 367–375.