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Amaranth proteins as a source of antioxidant peptides: Effect of proteolysis

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ABSTRACT

The antioxidant activity of peptides present in the phosphate buffer-soluble fraction of: – *Amaranthus mantegazzianus* protein isolates (*Is*), – protein fractions (*Albs*, *Globs*, *GlobPs* and *Gluts*), alcalase hydrolysates of isolates (hydrolysis degree –HD-: 2.4% (IH_{Is}) and 30% (IH_{hs}) and protein fractions (*AlbHs*, *GlobHs*, *GlobPHs*, and *GlutHs)* was investigated. Fractions separated by molecular exclusion chromatography were also analyzed. $ABTS^{+\cdot}$ scavenging method showed the presence of antioxidant peptides in *Is*, *Albs*, *Globs*, and *Gluts*, being the last the one with the highest activity. No activity was detected in the *GlobPs*. After hydrolysis, the scavenging activity of all samples increased, especially at high HD. The *GlobPs* fraction presented the highest scavenging capacity after hydrolysis. Naturally-occurring peptides and polypeptides presented also the capacity to inhibit the linoleic acid oxidation, which was partially lost after hydrolysis. FPLC fractionation evidenced the appearance of <0.5 kDa active peptides due to the hydrolysis process. Results suggest the presence in the *Is* and IH_{hs} of several peptides and polypeptides which can act as antioxidants by different mechanisms.

1. Introduction

Diverse metabolic pathways produce radical species (Halliwell & Gutteridge, 1986), while free radicals are used in various specific physiological functions. Free radicals in biological systems include reactive oxygen species (ROS) such as $O_2^{\cdot-}$, $HO_2^{\cdot}$, H_2O_2 and $OH^{\cdot}$, and reactive nitrogen species (RNS). Besides, the human body has diverse defense antioxidant mechanisms that can act at different levels of the oxidation process. The most important defense mechanisms are antioxidant enzymes (e.g. superoxide dismutase, catalase), free radicals scavengers (e.g. glutathione, vitamin C, vitamin E), and metal chelators (e.g. transferrin, ferritin) (Noguchi, Watanabe & Shi, 2000). When free radicals are generated in excess or when cellular defenses are deficient, biomolecules are damaged by a process named oxidative stress (Mendis, Kim, Rajapakse & Kim, 2007). This process seems to be implicated in cellular aging and also in associated diseases such as atherosis, cardiovascular disease, cancer, neurological degenerative diseases, and others (Wei & Lee, 2002).

Due to the negative consequences of the oxidative processes, their inhibition both inside the organism and in foods is important, this being an area of very intense research. Due to the current tendency to consume natural products and because of the demonstrated carcinogenic activity of the common synthetic antioxidants, there is a high demand of antioxidants of natural origin (Lindberg Madsen & Bertelsen, 1995). Vegetables contain numerous antioxidant compounds such as polyphenols, phytosterols, carotenoids, vitamins C and E, among others. While some proteins and protein hydrolysates have been also shown to posses antioxidant activity, this type of antioxidant compounds has been much less analysed. Antioxidant activity has been demonstrated in peptides derived from soy proteins (Chen, Muramoto, Yamauchi & Nokihara, 1996), caseins (Hernández-Ledesma, Dávalos, Bartolomé & Amigo, 2005; Pihlanto, 2006; Rival, Boeriu & Wichers, 2001), soy and milk whey (Peña-Ramos & Xiong, 2003), egg-yolk proteins (Sakanaba & Tachibana, 2006), just to mention some examples.

Amaranth, which belongs to the *Amaranthaceae* family, is an ancestral American crop that was used by Mayas, Aztecs and Incas and then prohibited after the conquest. It is considered as a pseudocereal and has a high nutritive value, with high protein content (15–17%) and an excellent aminoacidic balance. In addition, it can grow in adverse conditions such as dryness, high temperatures and saline soils, becoming an interesting crop especially in poor regions. The presence in amaranth seeds of some phytochemicals e.g. lecitines, polyphenols, saponins, trypsin inhibitors, phytates with physiological effects on humans has been described (Guzman-Maldonado & Paredes López, 1998, (chap. 9)). Besides, some biological activities of amaranth proteins have been demonstrated. The cholesterol-reducing effect of diets based on amaranth seeds or extruded amaranth was attributed to the presence of soluble fiber and proteins in these preparations (Plate & Arêas, 2002). Antimicrobial peptides were found in some amaranth species (Lipkin et al., 2005). Regarding the antioxidant properties of amaranth, such activity has been attributed to polyphenolic

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compounds and squalene present in the plant (Amin, Norazaidah & Hainida, 2006; Conforti, Statti, Loizzo, Sachetti & Poli, 2005; Nsimba, Kikuzaki & Konishi, 2008). However, the antioxidant activity of proteins or peptides from amaranth has not been yet studied.

The aim of the present work was to analyse the presence of proteins and/or peptides with antioxidant activity in seeds from *Amaranthus manteggazianus.* In addition, the potential improvement of the antioxidant activity by means of protein hydrolysis was also evaluated.

2. Materials and methods

2.1. Chemicals

Alcalase 2.4 L (protease of *Bacillus licheniformis*, Novozyme Corp), butylated hydroxytoluene (BHT), linoleic acid, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the other reagents were of analytical grade.

2.2. Samples

2.2.1. Protein isolates

Amaranthus mantegazzianus from commercial variety (*Pass cv Don Juan)* was grown at the Facultad de Agronomía, Universidad Nacional de La Pampa, Argentina, during the years 2006 and 2007. Flours corresponding to each year crop were obtained by grinding the whole seeds in an Udy mill, 1 mm mesh, screened by 0.092 mm mesh, and defatted by extraction with hexane during 24 h at 4 °C. Amaranth protein isolates (*I*) were obtained from the defatted flour by extraction at pH 9, isoelectric precipitation (pH 5), neutralization and freeze-drying (Martinez & Añón, 1996). Isolates obtained from seeds harvested in 2006 and 2007 presented similar protein content by Kjeldhal method: 78.2 ± 0.2 and 78.0 ± 0.5% w/w (dry base), respectively.

2.2.2. Protein fractions

Protein fractions – albumins (*Alb*), globulins (*Glob*), globulins P (*GlobP*) and glutelins (*Glut*) were obtained from defatted flour by sequential extraction followed by isolelectric precipitation and centrifugation (9000*g*, 20 min, 20 °C), neutralization, and freeze-drying, according to Martinez and Añón (1996), with some modifications. The *Alb* fraction was extracted from defatted flour with water (ratio 10 ml water/1 g flour) and precipitated by adjusting the pH of the dispersion to five. Then *Glob* were extracted by treating the resulting pellet with 32.5 mM K_2HPO_4/2.6 mM KH_2PO_4 (pH 7.5) containing 0.4 M NaCl, followed by precipitation as explained for the Alb fraction. *GlobP* were extracted by treatment of the last pellet with water, and subsequent precipitation by adjusting to pH 6. Finally, *Glut* were obtained from the resulting pellet by extraction with 0.1 M borate buffer (pH 10) and precipitation at pH 6. The whole process was performed at room temperature.

2.2.3. Protein hydrolysates

Suspensions (1% w/v) from *I*, *Alb*, *Glob*, *GlobP*, and *Glut* in 1 mM NaOH were prepared adjusting to pH 10. Suspensions were agitated during 1 h at 37 °C, maintaining the pH 10 by adding 0.1 M NaOH. After that, alcalase (⩾2.4 U/g, Anson Units) was added in a ratio of 8 μl/100 mg sample for high extent hydrolysates (*IHh*) or 0.08 μl/100 mg sample for low extent hydrolysates (*IHl*). Reaction mixtures were incubated at 37 °C during different times. The enzyme activity was stopped by heating at 85 °C during 10 min and suspensions were freeze-dried, obtaining the corresponding hydrolysates: *IHl*, *IHh*, *AlbH*, *GlobH*, *GlobPH* and *GlutH*. The hydrolysis grade (HD) was measured by reaction of free amino groups with 2,4,6-trinitrobencenesulfonic acid (TNBS) (Adler-Nissen, 1979) and calculated using the equation: $HD = ([-NH_2]h - [-NH_2]_0)/([-NH_2]_\infty - [-NH_2]_0) \times 100$; where $[-NH_2]$ indicates the concentration of free amino groups in the non-hydrolysed (0) or the hydrolysed sample (h). The parameter $[-NH2]_\infty$ was estimated according to: $[-NH2]_{\infty=}1/M_{aa} \times (1 + f_{Lys}) \times C_{prot}$, where M_{aa} is the average of the amino acid molecular weight in the amaranth proteins (130 g/mol), f_{Lys} is the rate of lysine in this proteins (1/15) (Bressani, 1994), and C_{prot} is the protein concentration (1 g/L for this assay).

2.3. Soluble fractions

Suspensions (10 mg/ml) from each freeze-dried sample (isolates, protein fractions and their corresponding hydrolysates) in 32.5 mM K_2HPO_4/2.6 mM KH_2PO_4 (pH 7.8) were prepared by vortexing and agitation during 1 h at room temperature. Suspensions were centrifuged at 92,000×*g* for 20 min at room temperature (Spectofuge 24D, Lab Net International) and supernatants were separated to obtain the corresponding soluble fractions. Soluble protein concentration was determined by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951). These samples were identified as: *Is*, *Albs*, *Globs*, *GlobPs*, *Gluts, IHls*, *IHhs*, *AlbHs*, *GlobHs*, *GlobPHs* and *GlutHs*.

2.4. Ultrafiltration

Fractions containing only soluble peptides with molecular mass <3 kDa ($Is_{3\_}$ and $IHs_{3\_}$) were obtained by filtration of *I* and *IHh* soluble fractions through a membrane (cut-off: 3 kDa) using an Amicon 810 system (Millipore Corp., Bedford, USA).

2.5. Chromatographic separation

Soluble total fractions in 35 mM phosphate buffer, pH 7.8 (obtained from 15 mg/ml suspensions) were analyzed in a Pharmacia LKB, FPLC System (Uppsala, Sweden), using a molecular exclusion Sepharose column (cut-off: 10 kDa) at room temperature. The exclusion volume was 8.0 ml, and elution volumes for standard peptides were: aprotinin (6.5 kDa): 14.2 ml; Tyr-Gly-Gly-Phe-Met (573.7 kDa) + Tyr-Gly-Gly-Phe-Leu (555.6 kDa): 18.7 ml; Val-Tyr-Val (379.5 kDa) + Gly-Tyr (238.2 kDa): 20.2 ml. Soluble samples (200 μl) were eluted with the buffer mentioned above. Polypeptides and peptides were detected by absorbance at 280 nm. Fractions corresponding to each peak were collected. Protein concentration in the fractions was determined by the Lowry method as modified by Stoscheck (1990) in order to reach a high sensitivity.

2.6. Antioxidant activity

Antioxidant activity was determined by two methods:

2.6.1. Scavenging of $ABTS^+$ radical

ABTS radical cation ($ABTS^+$) decoloration assay was performed according to Siddhuraju (2006) with some modifications. ABTS was dissolved in water to a 7 mM concentration. $ABTS^+$ was produced by reaction of ABTS solution with 2.45 mM potassium persulfate (final concentration) in the dark at room temperature for more than 16 h before use. Prior to the assay, the solution was diluted in water and equilibrated at room temperature to give an absorbance of 0.70 ± 0.02 at 734 nm in a 1 cm cuvette with a Beckman DU 650 spectrophotometer. Different volumes of sample (10–200 μl) were mixed with 1 ml of $ABTS^+$ solution, and absorbance at 734 nm was measured at different times. Appropriate solvent

blanks (negative controls NC) were run for each assay; while BHT was used as positive control (PC). Scavenging % was calculated as follows:

$$\text{Scavenging } \% = [(\text{Abc}_0 - \text{Abc}_t) - (\text{Abs}_0 - \text{Abs}_t)/\text{Abc}_0] \times 100$$

where: Abc_t and Abs_0 = absorbance of the negative control at $t = t$ and $t = 0$, respectively; Abs_t and Abs_0 = absorbance of the sample at $t = t$ and $t = 0$, respectively. Scavenging % was plotted as a function of the mass of protein contained in the sample in order to obtain the mass of protein necessary to reduce absorbance by 50% (IC50).

2.6.2. Inhibition of the linoleic acid oxidation

Inhibition of oxidation was measured in a linoleic acid emulsified system ((Liu, Chen & Lin, 2005). For this purpose, an emulsion of 10 mM linoleic acid in 35 mM phosphate buffer (pH 7.8) with 0.5% w/v polyoxyethylenesorbitan monolaurate was prepared by homogenization in Ultraturrax (14,000 rpm, 30 seg). Equal volumes of emulsion and sample were then mixed and incubated at 50 °C. Negative control NC (buffer) and positive control PC (Trolox) were run in parallel. The oxidation level was measured in aliquots obtained at different times of incubation using the ferric thiocianate method (Sakanaba, Tachinaba, Noriyuki & Juneja, 2004). Briefly, 50 µl of reaction mixture, 2.35 ml of 75% ethanol, 50 µl of 30% ammonium thiocyanate and 50 µl of 20 mM ferrous chloride solution in 3.5% HCl were mixed, and after 3 min the absorbance of the colored solution was measured at 500 nm. Percent inhibition of the oxidation was calculated as:

$$\text{Inhibition } \% = 100 \times [1 - (\text{Abs}_t - \text{Abs}_0)/(\text{Abc}_t - \text{Abc}_0)]$$

where Abs_t and Abs_0 = sample absorbance at $t = t$ and $t = 0$, respectively; Abc_t and Abs_0 = negative control absorbance at $t = t$ and $t = 0$, respectively.

3. Results and discussion

3.1. Characterization of samples

As previously mentioned the objective of this work was to analyze the presence of antioxidant activity in peptides naturally present in *Amaranthus mantegazzianus* seed as well as in those generated by enzymatic hydrolysis of the seed proteins. For that, only the fractions soluble in 35 mM phosphate buffer pH 7.8 – *Is*, *Albs*, *Globs*, *GlobPs*, *Gluts, IHls*, *IHhs*, *AlbHs*, *GlobHs*, *GlobPHs* and *GlutHs*.- containing polypeptides and peptides of relatively low molecular weight were analyzed.

3.1.1. Molecular exclusion chromatography (FPLC)

According to our objective, polypeptide and peptide composition of the studied samples was analyzed by FPLC using a molecular exclusion column with a cut-off = 10 kDa, which allows to analyze low molecular weight molecules. Fig. 1 shows the chromatogram corresponding to the *Is* fraction. Estimated molecular mass ranges corresponding to each peak are presented in Table 1. There was a significant presence of peptides with molecular mass lower than 10 kDa in *Is* (about a 78% of the total protein mass), including very small peptides (about a 3.5% of the total protein mass) (Table 5). The polypeptide composition of the *Is* fraction cannot be compared with those currently described for amaranth isolates (Martinez & Añón, 1996, Martínez, Castellani and Añón 1997, Scilingo, Molina-Ortiz, Martínez and Añón, 2002) because it contains only proteins and polypeptides soluble in phosphate buffer at pH 7.8.

Proteins were separated according to their solubility characteristics in *Alb*, *Glob*, *GlobP* and *Glut* fractions which presented the usual polypeptide composition corresponding to amaranth storage proteins (data not shown). Phosphate buffer-soluble components from each fraction were analyzed by FPLC and compared with those present in *Is*. Fig. 2 shows the corresponding chromatograms; and the presence of these fractions in the chromatographic peaks of *Is* is shown in Table 1. *Albs* fraction components were present in all peaks of the *Is* FPLC profile, thus making *Albs* the most widely distributed fraction (Fig. 2a). *Globs* components were present in most peaks except peak 5 (Fig. 2b), while *GlobPs* components appeared only in peaks 1 and 5 (Fig. 2c). *Gluts* fraction presented only components of molecular weight > 10 kDa and others of very low molecular weight (<0.5 kDa) (Fig. 2d). In addition, a new peak (peak 8) at high elution volume corresponding to a molecular mass < 0.25 kDa, which was not detected in the *Is* chromatogram, appeared mainly in the *Albs* fraction (Fig. 2a) and to a lower extent in the *Gluts* fraction (Fig. 2d).

Hydrolysates. In order to release peptides encrypted in amaranth proteins, the isolate *I* was subjected to hydrolysis with alcalase. Two different preparations were obtained according to the extent of the hydrolysis: -*high hydrolysis grade* (*IHh*), and -*low hydrolysis grade* (*IHl*). The latter was obtained after 10 min of reaction of *I* with 0.08 µl alcalase/100 mg, while *IHh* was obtained after 4 h of reaction under the same conditions. The corresponding hydrolysis grades (HG) are shown in Table 2. In addition, hydrolysis products soluble in phosphate buffer pH 7.8 were characterized by molecular exclusion FPLC and compared with *Is* chromatogram. As shown in Fig. 3, *IHls* presented polypeptides with molecular mass >10 kDa as demonstrated by a decrease of peak 1, and peptides of very low molecular mass (elution volume > 22 ml) resulting from the

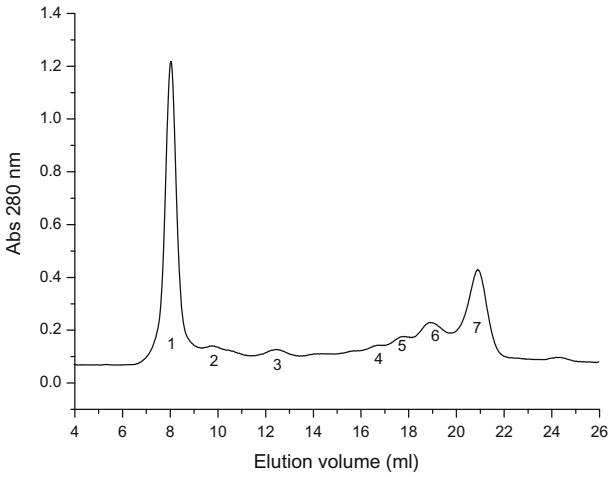

Fig. 1. FPLC molecular exclusion chromatogram of soluble fractions of *Is*, indicating the peaks (1–7) with their corresponding elution volumes.

Table 1
Molecular mass of FPLC peaks from *I* and constituent protein fractions.

Peak *N*° in *I*	Mass range	Protein mass (µg protein)	Component fractions
1	>10	520	*Alb*, *Glob*, *Glut* ≫ *GlobP*
2	10–6.5	55	*Glob* ≫ *Alb*
3	10–6.5	78	*Alb*
4	6.5–0.5	33	*Glob*
5	6.5–0.5	2	*Alb*, *GlobP* > *Glut*
6	⩽0.5	nd	*Alb*, *Glob*
7	<0.5	25	*Alb*, *Glob* ≫ *Glut*
8[a]	<0.25	–	*Alb*, *Glut*

nd: non detected.
[a] Peak 8 was not detected in *I* chromatogram.

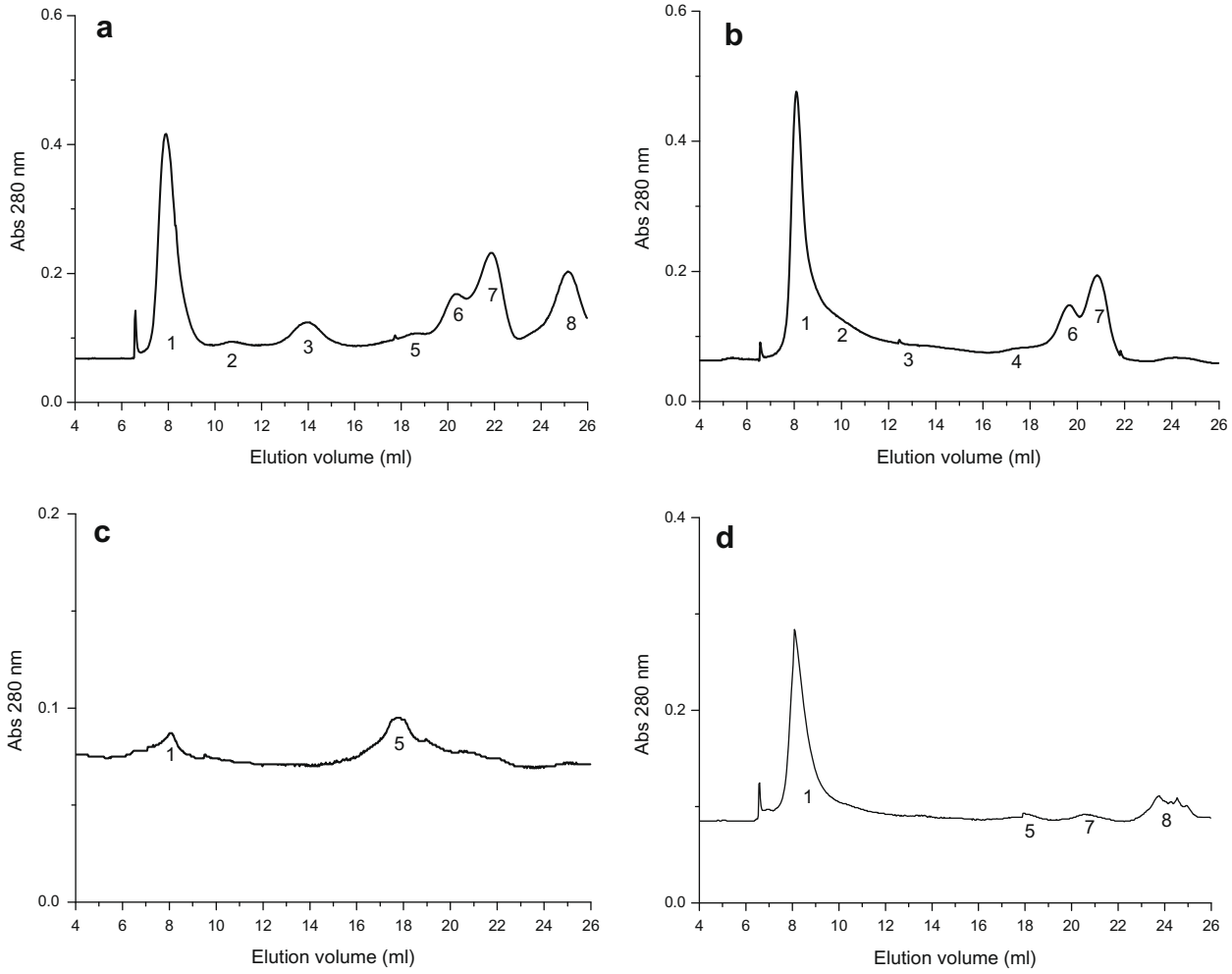

Fig. 2. FPLC molecular exclusion chromatograms of soluble components from amaranth protein fractions: (a) *Albs*, (b) *Globs*, (c) *GlobPs*, and (d) *Gluts*.

Table 2
Hydrolysis grade of amaranth isolate and protein fractions treated with alcalase determined by the TNBS method.

Sample	HG %
IH_h	29.1 ± 1.0
IHl	2.4 ± 0.04
AlbH	19.3 ± 1.8
GlobH	16.4 ± 0.4
GlobPH	20.9 ± 0.2
GlutH	20.2 ± 0.8

Results are shown as the mean ± SD of two determinations.

hydrolysis reaction. In contrast, *IHhs* presented an almost total hydrolysis of species with mass > 10 kDa and those between 10 and 6.5 kDa (peaks 1, 2, 3 and 4), with a higher intensity of peptides of low molecular mass (Fig. 3). Thus, according to protein quantification data, *IHhs* was constituted by a very high proportion of peptides with molecular mass < 0.5 kDa (about a 47% of the total protein mass), including a significant presence (2.6%) of molecules of very low molecular mass (<0.25 kDa).

Based on previous results and because of our interest in small peptides, protein fractions were hydrolysed to obtain a high HG (4 h, 8 μl alcalase/100 mg sample) (Table 2). Although similar hydrolysis conditions were used in all cases, all protein fractions were hydrolysed in a lesser extent than *I* sample. *Alb*, *GlobP*, and *Glut* fractions showed similar HD, slightly higher than that of the *Glob* sample.

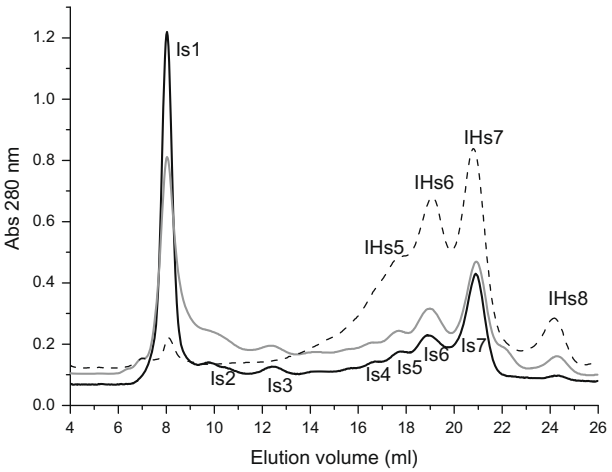

Fig. 3. FPLC molecular exclusion chromatograms (cut-off: 10 kDa) corresponding to amaranth *Is* (—), *IHls* (—), and *IHhs* (—).

3.1.2. Protein solubility

The protein solubility of each freeze-dried sample in 35 mM phosphate buffer (pH 7.8) was determined by the Lowry method. For this purpose, soluble fractions were obtained by centrifugation of suspensions prepared adding 10 mg of freeze-dried sample per

Table 3
Protein solubility in 35 mM phosphate buffer, pH 7.8 of amaranth isolate and protein fractions before and after hydrolysis with alcalase.

Sample	Solubility % (g protein/100 g freeze-dried sample)	
	Non-hydrolysed	Hydrolysed
I	74.4 ± 2.5	98.2 ± 4.8
Alb	46.6 ± 1.8	84.5 ± 0.9
Glob	29.9 ± 1.4	57.8 ± 0.5
GlobP	10.8 ± 0.2	98.6 ± 0.9
Glut	6.5 ± 1.1	57.9 ± 1.0

Results are shown as the mean ± SD of two determinations.

ml of buffer. Results are shown in Table 3. The low solubility of *GlobP* and *Glut* fractions explains the poor contribution of these fractions in the FPLC chromatograms; this fact being in agreement with the solubility characteristics of these protein fractions (Segura-Nieto, Barba de la Rosa & Paredes-López, 1994, chap. 5). Due to our interest in small peptides with antioxidant activity, a fraction containing only peptides with a molecular mass lower than 3 kDa (Is_{3-}) was obtained by ultrafiltration of the soluble fraction of *Is*. Protein determination in Is_{3-} fraction showed that about 16% of the total mass of freeze-dried *I* (16.2 ± 0.8 g/100 g *I*) or, in other way, 21.8% of *Is* total proteins (16.2 g/74.4 g), corresponded to < 3 kDa species.

Studies on the protein solubility of the freeze-dried hydrolysates in 35 mM phosphate buffer (pH 7.8) revealed a notable increase of this parameter due to the hydrolysis process (Table 3). When *IHhs* was ultrafiltrated by a membrane with cut-off = 3 kDa (yielding fraction IHh_{3-}) almost 80% of proteins were filtrated (78.1 ± 0.2 g/100 g *I* or 78.1/98.2 g *Is* proteins), confirming the high proportion of species of low molecular mass. In all cases, the solubility of hydrolysed protein fractions was notably increased as compared to the corresponding non-hydrolysed fractions (Table 3), this change being more marked for the *GlobP* fraction.

3.2. Antioxidant activity of soluble samples

3.2.1. Isolate, protein fractions and effect of proteolysis

The scavenging activity of the *Is* fraction was determined by the ABTS$^+$ radical method. Preliminary assays demonstrated that radical absorbance was stabilized after 10 min of reaction with samples or known antioxidant compounds, as shown in Fig. 4a for *Is* samples with different protein concentrations. Thus, 10 min of reaction was selected as the measurement time to obtain the scavenging % and the IC_{50} value from the corresponding plot (scavenging % as a function of protein mass). An IC_{50} value of 93.5 μg of protein was obtained for *Is* derived from the 2006 amaranth crop, while the corresponding value for the *Is* from the 2007 amaranth crop was 91.3 μg of protein (Fig. 4b). These results showed a comparable scavenging capacity for both isolates, with a mean value of 92.4 ± 1.6 μg of protein. On the other hand, the IC_{50} value for BHT was 3.7 μg, which can be used to compare the antioxidant potency of the analyzed samples.

To determinate the contribution of species with different molecular mass to the scavenging capacity of *Is*, the Is_3 fraction activity was measured. The IC_{50} value corresponding to this fraction was 45.2 μg of protein (Fig. 4b), approximately 50% lower than the corresponding IC_{50} for *Is*, indicating a higher scavenging capacity of the fraction of low molecular weight. This fact would be related to two effects of the filtration process: the concentration of the more active molecules and the elimination of non-antioxidant and/or pro-oxidant molecules.

The ABTS$^+$ scavenging activity of each soluble protein fraction (*Albs*, *Globs*, *GlobPs*, and *Gluts*) was also analyzed. The corresponding IC_{50} values for *I* and protein fractions are shown in Table 4. The scavenging capacity presented the following order: *Gluts* > *Globs* > *Albs*. In the case of the *GlobPs* fraction, no activity was registered up to a protein mass of 114 μg in the reaction mixture. Samples containing a higher protein amount were difficult to assay due to the very low solubility of this fraction, as was previously mentioned. The *Gluts* fraction presented a higher scavenging capacity than *Is,* probably related to the presence of very low molecular weight species in this fraction as revealed by FPLC or, may be, to the presence of molecules with high activity. On the other hand, the *Albs* fraction containing also small peptides showed a low activity, which may be due to the presence of a high proportion of molecules of high molecular weight or the presence of less active molecules.

The effect of protein hydrolysis on the ABTS$^+$ scavenging activity was analyzed. Fig. 5 shows the results obtained for *IHls* and *IHhs* as compared to *Is*. As shown in Table 4, IC_{50} values were 92.4 μg, 74.1 μg and 27.8 μg of protein for *Is*, *IHls,* and *IHhs*, respectively, demonstrating a positive effect of protein hydrolysis on the scavenging capacity of the samples. In particular, an extensive hydrolysis produced a peptide mixture of very high activity. When *IHhs*

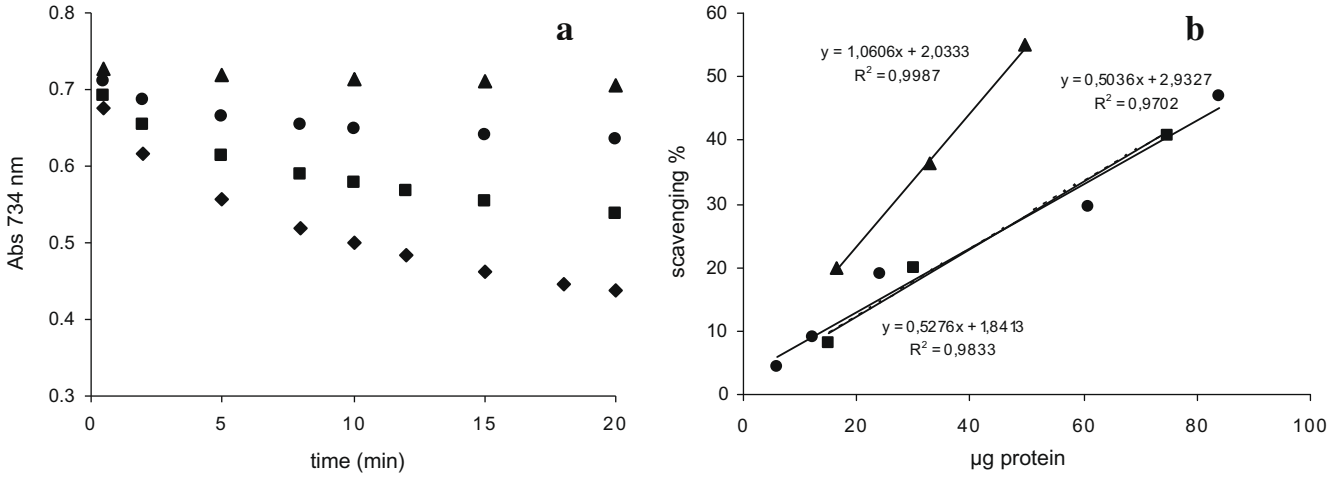

Fig. 4. ABTS$^+$ scavenging activity f *I* samples. (a) Absorbance decay as a function of time for *Is* samples (10 μl) with different protein concentration: ▲ NC, ● 1.2 μg/μl, ■ 2.4 μg/μl, ♦ 6.1 μg/μl. (b) Scavenging % as a function of protein amount for: ● *Is* (2006), ■ *Is* (2007), ▲ Is_{3-}; linear regression equations shown for each curve were used to calculate the corresponding IC_{50} values.

Table 4
IC_{50} values of ABTS assay for isolate and protein fractions before and after hydrolysis with alcalase.

Sample	IC_{50} (ug protein)	
	Non-hydrolysed	Hydrolysed
I	92.4	74.1 (*IHl*) 27.8 (*IHh*)
$I_{3\_}$	45.2	28.6 ($IHh_{3\_}$)
Alb	423.1	35.6
Glob	127.1	45.8
GlobP	nd[a]	16.5
Glut	25.1	22.4

[a] No activity was detected with doses up to 114 μg of proteins.

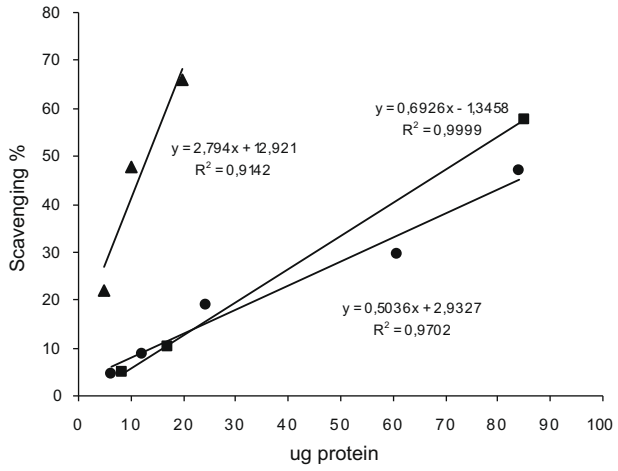

Fig. 5. Scavenging % as a function of protein amount for: ● *Is*, ■ *IHls*, ▲ *IHhs*; linear regression equations shown for each curve were used to calculate the corresponding IC_{50} values.

was ultrafiltrated to obtain $IHhs_{3\_}$ the IC_{50} value did not change (Table 4), in agreement with the very high proportion of low molecular weight molecules in the hydrolysate. Soluble fractions of high extent hydrolysates from the different amaranth protein fractions were also analyzed. As shown in Table 4, hydrolysis increased the scavenging activities of all fractions, including the *GlobPHs* fraction which had not exhibited such activity before hydrolysis but showed the lowest IC_{50} value. *GlutHs* did not present important modifications respect to the non-hydrolysed soluble fraction. In summary, the scavenging capacity of fractions after hydrolysis was *GlobPHs* > *GlutHs* > *AlbHs* > *GlobHs*.

Wang and Xiong (2005) have demonstrated that peptides with antioxidant activity in hydrolysed potato proteins were mostly small molecules which were preferred targets of free radicals. However, a bigger antioxidant protein (about 28 kDa) was detected in water extracts of Sundakai (*Solanum torvum*) seeds, presenting good linoleic acid antioxidant, free radical scavenging, reducing, and chelating activities (Sivapriya & Srinivas, 2007). These facts agree with our finding of scavenging activity due to molecules of diverse molecular weights in amaranth *Is* and *Ihs* fractions.

3.2.2. Soluble fractions separated by FPLC

As a first approach to the isolation of peptides and polypeptides with antioxidant activity, fractions corresponding to each peak of the *Is* and *IHhs* FPLC chromatograms were collected. The $ABTS^+$ scavenging activity was determined on these fractions. Determinations were performed using different volumes of each fraction (100, 150 and 200 μl). Scavenging % obtained with 150 μl of sample and IC_{50} values are shown in Table 5. In some cases, such as Is_2, Is_3, Is_4, and Is_7 fractions, the scavenging % measured were low because of the low protein concentration of the fractions. In these cases, IC_{50} values were obtained by extrapolation of this activity at low inhibition % values. In the case of Is_5 and Is_6 fractions, protein concentrations were below the detection limit of the protein determination method. While these fractions presented some scavenging activity, IC_{50} values could not be calculated.

In the case of the *IHhs* sample, fractions that were modified by the hydrolysis process were analyzed: IHs_5, IHs_6, IHs_7 and IHs_8. IC_{50} values are shown in Table 5. Comparing *Is* and *IHhs* samples, fractions 5, 6 and 7 showed a marked increase in the scavenging % of a constant volume of sample. This increment would be related to the increase in the peptide concentration of these fractions, indicating the appearance of new small active molecules. However, in the case of IHs_7 the IC50 value was similar to those corresponding to Is_7, suggesting a similar specific activity of the new peptides respect to those present in the non-hydrolysed isolate. In the case of IHs_8 fraction, scavenging % was low and, in addition, it was not possible to obtain a linear relationship between the scavenging activity and the protein amount in the sample.

Antioxidant activity was also assayed by a different methodology, which reflects the capacity of a sample to inhibit the oxidation of the linoleic acid. The antioxidant activity of amaranth isolate and peptides has been previously demonstrated using this methodology (Tironi & Añón, 2007). In addition, when activity of isolate (*Is*) and hydrolysates (*IHls* and *IHhs*) were compared,

Table 5
Antioxidant activity of FPLC fractions from *I* and *IHh*.

Sample	FPLC fraction	Estimated MW (kDa)	Protein conc. (μg/μl)	$ABTS^+$ assay		Linoleic acid	
				Scavenging % (150 μl)	IC_{50} (μg prot)	%inhib	%inhib/μg protein
I	I_1	>10	0.2	37.2	46.4	4.0	0.08
	I_2	6.5–10	0.029	8.1	32.2	91.5	12.6
	I_3	6.5–10	0.041	14.5	21.5	2.1	0.2
	I_4	0.5–6.5	0.037	3.1	42.8	nm	nm
	I_5	0.5 a 6.5	nd	3.4	–[a]	16.2	–[a]
	I_6	<0.5	nd	4.2	–[a]	31.8	–[a]
	I_7	<0.5	0.011	4.7	15.9	36.8	13.4
IHh	IH_5	0.5 a 6.5	0.31	66.8	32.9	10.5	0.14
	IH_6	<0.5	0.31	83.0	21.0	35.1	0.45
	IH_7	<0.5	0.046	27.9	15.3	40.0	3.5
	IH_8	<0.25	0.017	5.6	–[b]	45.1	10.4

nd: non detectable.
nm: not measured.
[a] Value could not be calculated because of protein concentration was undetectable.
[b] Value could not be calculated because of non lineal relation dose–response was obtained.

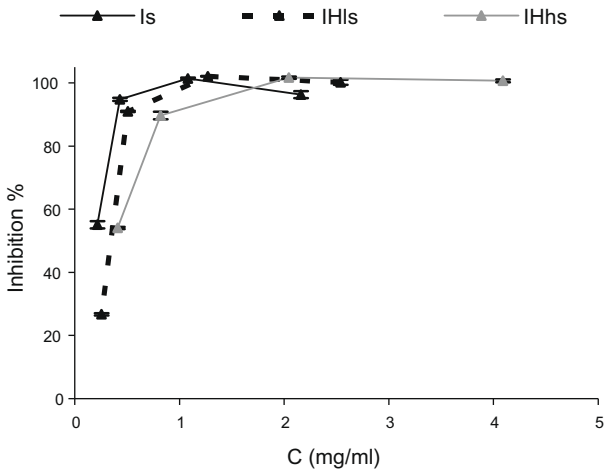

Fig. 6. Linoleic acid oxidation inhibition assay: oxidation level (absorbance at 500 nm in the ferric thiocianate assay) after 24 h of incubation in the presence of *Is*, *IHIs*, and *IHhs*.

results were different of that obtained by the scavenging method. All the samples presented antioxidant activity by this methodology (oxidation level is measured by the ferric thiocyanate assay (absorbance at 500 nm)), being this activity dependent on the protein concentration up to achieved 100% inhibition (Fig. 6). However, hydrolysis process produced a shift of dose–response curves to the right, indicating that more polypeptides are necessary to obtain a similar level of inhibition of the oxidation. Thus, hydrolysis produced a decreased on the antioxidant capacity by this methodology, suggesting that at less part of this capacity were due to proteins or large polypeptides present in the isolate and then hydrolyzed.

Taking these antecedents into account, we decided to evaluate the ability of the FPLC fractions to inhibit linoleic acid oxidation. The antioxidant Trolox (5 μM) was used as a positive control (PC). Results are shown in Fig. 7. The oxidation level was maximal after 18 h of incubation, as can be seen for the negative control (NC), in which the sample was replaced by 35 mM phosphate buffer, pH 7.8. In contrast, no changes in the oxidation level were observed during the incubation time in the tube containing the PC. For FPLC fractions from *Is* (Fig. 7a), the most active fraction was Is_2 which presented an inhibition of 91.5% at 18 h of incubation (Table 5). Also, Is_7, Is_6 and Is_5 fractions showed activity, with 36.8%, 31.8% and 16.2% inhibition, respectively. When activity was expressed per μg of protein present in the fraction, Is_2 and Is_7 resulted the most active fractions; although Is_5 and Is_6 probably have high activities taking into account their very small amount of peptides (not detectable) (Table 5). In the case of *IHhs* FPLC fractions (Fig. 7b), IHs_8, IHs_7 and IHs_6 presented important activity with 45.1%, 40.0%, and 35.1% inhibition of the linoleic acid oxidation, while it was minor for IHs_5 (10.5%) (Table 5). When activity was expressed per μg of protein, IHs_8 presented the higher value. It is important to remark that if similar mass range fractions of *Is* and *IHs* are compared, although the protein concentration of fractions 5, 6 and 7 notably increased due to the hydrolysis process, the inhibition % decreased (fraction 5) or presented a very slight increment (fractions 6 and 7). These facts suggest that peptides produced by hydrolysis would have a minor potency to inhibit the oxidation of linoleic acid in agreement with results obtained with the complete *IHhs* fraction. In other way, IHs_8 fraction produced a high inhibition of linoleic acid oxidation, but had a poor scavenging activity suggesting a mechanism of antioxidation different from free radical scavenging, as was previously suggested by the results obtained for *Is* and *IHs* samples.

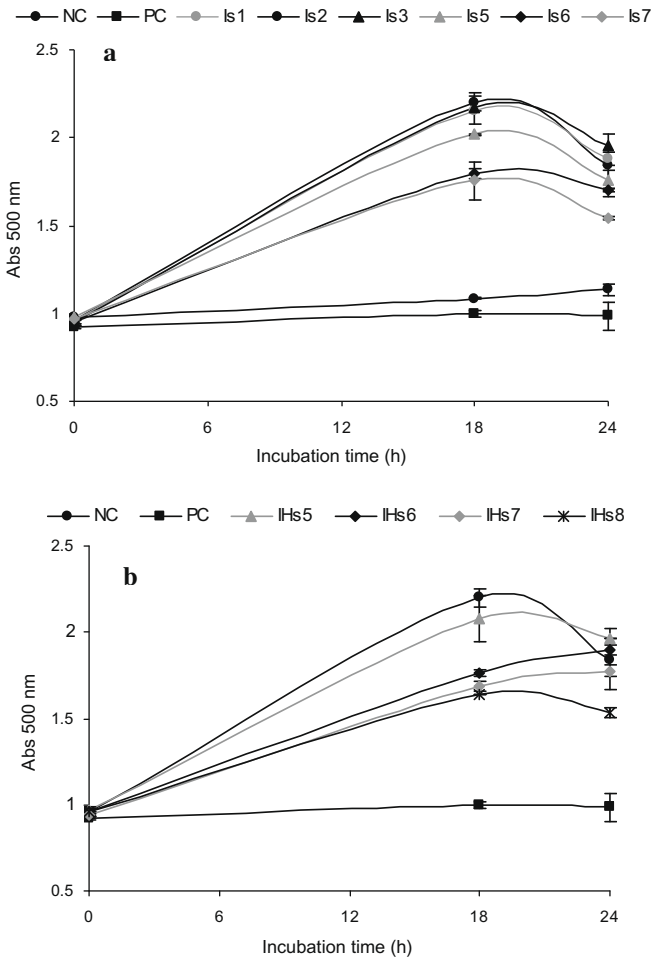

Fig. 7. Linoleic acid oxidation inhibition assay. (a) FPLC fractions from *Is*, (b) FPLC fractions from *IHhs*. NC: negative control (buffer); PC: positive control (Trolox 5 μM).

4. Conclusions

This study demonstrated the presence in the *Amaranthus mantegazzianus* seeds of naturally-occurring peptides and polypeptides, soluble in phosphate buffer pH 7.8, which possess free radical scavenging activity. Active molecules were distributed into the different protein fractions (Albs, Globs and Gluts), being the Gluts fraction the one with the highest activity. In addition, alcalase hydrolysis was able to improve the scavenging activity of both the isolate and the protein fractions by producing the release of small peptides and/or free amino acids with such activity. The GlobPs fraction which was not active before the hydrolysis – presented the highest scavenging capacity after the hydrolysis process. Molecules with molecular weight <0.5 kDa showed to be the most active ones. Hydrolysis produced an increment in the scavenging activity of these fractions due to an increase in the amount of peptides but not necessarily in the specific activity of the generated molecules. Naturally-occurring peptides and polypeptides presented also the capacity to inhibit the linoleic acid oxidation, which was partially lost after hydrolysis. However, the hydrolysis process generated a fraction with molecular weight <0.25 kDa which did not exhibit a good scavenging activity but had a considerable capacity of preventing the linoleic acid oxidation. Results suggest the presence in the amaranth isolate and alcalase-hydrolysate of several peptides and polypeptides which can act as antioxidants by different mechanisms. Although it is difficult

to compare the antioxidant capacity with the data existing in the literature due to the diversity of methodologies used, amaranth seems to be a good potential source of antioxidant peptides. Studies are under way in our laboratory to clarify the physiological significance of these molecules.

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