LWT - Food Science and Technology 44 (2011) 1752–1760

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Antioxidant activity of amaranth protein or their hydrolysates under simulated gastrointestinal digestion

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ARTICLE INFO

Article history:
Received 16 September 2010
Received in revised form
31 March 2011
Accepted 11 April 2011

Keywords:
Amaranth proteins
Simulated gastrointestinal digestion
Antioxidant peptides

ABSTRACT

Amaranth proteins were subjected to a simulated gastrointestinal digestion to evaluate the antioxidant activity of the products. A protein isolate (*I*) was first hydrolyzed with pepsin (Pe) (pH 2, 37 °C) and then with pancreatin (Pa) (pH 6, 37 °C). Different hydrolysis conditions were assayed and control reactions (without enzymes) were performed. Hydrolysis degree (HD) determined by TNBS method ranged from 13 to 37%. Soluble fractions in 35 mmol/L phosphate buffer, pH = 7.8 were obtained from freeze-dried samples, and antioxidant activity was evaluated by the $ABTS^{+}\cdot$ scavenging and the ORAC assays. Antioxidant activity increased significantly ($p < 0.05$) after simulated gastrointestinal digestion. According to the results, digestion conditions (Pe/protein: 1:10, 60 min; and Pa/protein: 1:10, 60 min) were selected and applied to an amaranth protein alcalase-hydrolysate (*H*) (HD = 29.2 ± 1.3). After pepsin and pancreatin action (*Hpepa*), HD was 42.0 ± 2.6, slightly higher than that of the digested isolate (*Ipepa*) (36.9 ± 0.5). The corresponding soluble fractions exhibited different electrophoretic profiles (tricine-SDS-PAGE) and gel filtration chromatograms, evidencing the presence of different molecular species. Previous hydrolysis with alcalase did not improve the antioxidant activity after simulated gastrointestinal digestion according to the methodologies assayed. Both the protein isolate and the alcalase-hydrolysate showed a potential capacity to scavenge free radicals after gastrointestinal digestion, appearing as promising ingredients to formulate functional foods with antioxidant activity.

1. Introduction

Food proteins may act as sources of bioactive peptides. These peptides are encrypted in the proteins and can be released *in vitro* during the food processing or *in vivo* by the gastrointestinal digestion. Bioactive peptides can exert their effects on the gastrointestinal system or be absorbed through the intestinal tract and act on important systems. Transepithelial oligopeptide transport by different routes has been studied (Satake et al., 2002). Recently, peptides with potential antimicrobial, antithrombotic, antihypertensive, cholesterol lowering, immunomodulatory, and antioxidant activities have been isolated and identified (Hartmann & Meisel, 2007).

Diverse metabolic pathways produce radical species (Halliwell & Gutteridge, 1986), including reactive oxygen species (ROS) such as $O_2\cdot^-$, $HO_2\cdot$, H_2O_2 and $OH\cdot$, and reactive nitrogen species (RNS). When free radicals are generated in excess or when cellular defences are deficient, biomolecules are damaged by a process called oxidative stress (Mendis, Kim, Rajapakse, & Kim, 2007). This process seems to be implicated in cellular ageing and also in associated diseases such as atherosis, cardiovascular disease, cancer, neurological degenerative diseases (Wei & Lee, 2002), as well as in gastrointestinal disorders (Ma, Xiong, Zhai, Zhu, & Dziubla, 2010). Due to the negative consequences of the oxidative processes their inhibition, both inside the organism and in foods, is of great importance. Some food proteins and protein hydrolysates have been reported to have antioxidant capacity. This 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), and buckwheat proteins (Ma et al., 2010), among others.

Amaranth is an ancestral American crop considered as a pseudo cereal. It has a high nutritive value, with high protein content (15–17 g/100 g) and an excellent aminoacidic balance (Segura-Nieto, Barba de la Rosa, & Paredes-López, 1994). In addition, it can grow in adverse conditions such as dryness, high temperatures, and saline soils, thus constituting an interesting crop especially in poor regions. The presence of some phytochemicals in amaranth seeds

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doi:10.1016/j.lwt.2011.04.002

with physiological effects on humans has been described (Guzman-Maldonado & Paredes.López, 1998). Besides, some biological activities of amaranth proteins have been demonstrated (Lipkin et al., 2005; Plate & Arêas, 2002). In a previous work, authors demonstrated the presence in *Amaranthus mantegazzianus* seeds of naturally-occurring peptides and polypeptides with free radical scavenging and inhibition of the linoleic acid oxidation activities. Active molecules were distributed into the different protein fractions (albumin, globulin and glutenin), the glutenin fraction being 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 (Tironi & Añón, 2010).

Peptides presenting radical scavenging capacity have been released by simulated gastrointestinal digestion from diverse food sources (Ma et al., 2010). In the present work, a simulation of the gastrointestinal digestion process was performed on two different amaranth products: a protein isolate and an extensive alcalase-hydrolysate. Antioxidant activity of the different preparations was analyzed in order to evaluate the potential formation of active peptides due to the gastrointestinal digestion.

2. Materials and methods

2.1. Chemicals

Pepsin 1:15000 5X NF standards and porcine Pancreatin 4X-100 USP units/mg were from MP Biomedicals LLC (Solon, OH, USA). About 2.4 L Alcalase (protease of *Bacillus licheniformis*, Novozyme Corp), butylated hydroxytoluene (BHT), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylicacid), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), Trizma base (Tris (hydroxymethyl) aminomethane), sodium dodecyl sulphate, tricine, bovine serum albumin, and RP-HPLC peptide mixture were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fluorescein sodium was from Fluka (Steinheim, Germany) and AAPH (2,2′-Azo-bis-(2-methylpropionamidine) dihydrochloride) was from Aldrich (Wisconsin, USA). All the other reagents were of analytical grade.

2.2. Samples

2.2.1. Protein isolates

A. mantegazzianus from commercial variety (*Pass cv Don Juan)* was grown at Facultad de Agronomía, Universidad Nacional de La Pampa, Argentina. Flour was 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 were obtained from the defatted flour by extraction at pH 9, isoelectric precipitation (pH = 5), neutralization and freeze-drying (Martinez & Añón, 1996). Isolates presented the following composition: 79.1 ± 0.1 g proteins/100 g, 9.7 ± 0.4 g carbohydrates/100 g; 7.0 ± 0.3 g water/100 g, 3.2 ± 0.1 g ash/100 g, and 1.7 ± 0.2 g lipids/100 g.

2.2.2. Alcalase hydrolysate

A 1 g isolate/100 mL suspension in 1 mmol/L NaOH was prepared and adjusted to pH = 10. Suspension was shaken for 1 h at 37 °C, maintaining the pH = 10 by adding 0.1 mol/L NaOH. After that, alcalase (≥2.4 U/g, Anson Units) was added at 8 μl/100 mg sample. The reaction mixture was incubated at 37 °C for 4 h to obtain an extensive hydrolysate (Tironi & Añón, 2010). The enzyme activity was stopped by heating at 85 °C for 10 min, and the suspension was freeze-dried.

2.2.3. Simulated gastrointestinal digestion

The method used was an adaptation of published methods (Hernández-Ledesma, Amigo, Ramos, & Recio, 2004; Roesler & Rao, 2001), and was optimized for the amaranth protein isolate. The protein isolate was initially treated with a pepsin solution (Pe) in 0.1 equi/L HCl and 0.03 mol/L NaCl (pH = 2) at 37 °C with agitation. Then, pH was adjusted to 6 and a pancreatin solution (Pa) in 0.1 mol/L $NaHCO_3$ (pH = 6) was added, incubating at 37 °C with agitation. Different pepsin/protein (1/20 and 1/10 g/g) and pancreatin/protein (1/100 and 1/10 g/g) ratios and reaction times (60 and 90 min for Pe, 30 and 60 min for Pa) were assayed. In all cases, control reactions, without enzymes added, were performed. Pancreatin activity was stopped by heating at 85 °C for 10 min and suspensions were freeze-dried.

The alcalase-hydrolysate was submitted to a similar simulated gastrointestinal digestion, using the optimal conditions selected from isolate assays. These conditions were: pepsin/protein ratio: 1/10 g/g, pepsin reaction time: 60 min; pancreatin/protein ratio 1/10 g/g, pancreatin reaction time: 60 min.

2.3. Hydrolysis degree determination

The hydrolysis degree (HD) was measured by reaction of free amino groups with 2,4,6-trinitrobencenesulfonic acid (TNBS) (Adler-Nissen, 1979) and calculated using the equation:

$$HD = \left([-NH_2]h - [-NH_2]_0\right)/\left([-NH_2]_\infty - [-NH_2]_0\right) \times 100;$$

where $[-NH_2]$ indicates the concentration of free amino groups in the non-hydrolysed (0) or the hydrolysed samples (h). The parameter $[-NH2]_\infty$ was estimated according to:

$$[-NH_2]_\infty = 1/M_{aa} \times \left(1 + f_{Lys}\right) \times C_{prot}$$

where M_{aa} is the average molecular weight of amino acids present in amaranth proteins (130 g/mol), f_{Lys} is the proportion of lysine in these proteins (1/15) (Bressani, 1994), and C_{prot} is the protein concentration.

2.4. Protein content of freeze-dried samples

MicroKjeldahl digests were analyzed by the colorimetric method of Nkonge and Balance (1982), with some minor modifications. Briefly, samples containing 5 mg of proteins were digested and then diluted to 25 mL with distilled water. A calibration curve was prepared by similar digestion of different amounts of bovine serum albumin (between 0 and 60 μg of nitrogen/mL solution).

2.4.1. Colorimetric reaction

Solution A (Na_2HPO_4 (0.20 mol/L), NaOH (0.20 mol/L), sodium potassium tartrate tetrahydrate (0.36 mol/L)) and solution B (2.50 mol/L NaOH) were mixed on a 1:l volume basis to prepare the working buffer. To 0.25 mL of the diluted digest, 0.75 mL of working buffer, 0.20 mL of salicylate-nitroprusside reagent (20 g sodium salicylate and 30 mg sodium nitroprusside in 100 mL) and 0.10 mL of the hypochlorite solution (5 g available chlorine/100 mL) were added. After agitation (20 min, room temperature) and dilution with distilled water, absorbance at 660 nm was measured. Protein content was calculated using a Kjeldahl factor of 5.85 (Becker et al., 1981).

2.5. Soluble fractions

10 mg/mL suspensions from each freeze-dried sample in 32.5 mmol/L K_2HPO_4/2.6 mmol/L KH_2PO_4 (pH = 7.8) were prepared by agitation at 300 rpm during 1 h at 37 °C (Termomixer

Eppendorf). Suspensions were centrifuged at 10,400× g for 10 min at room temperature (Spectrafuge 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), using bovine serum albumin as standard.

2.6. Tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis

Soluble fractions were analyzed by tricine-SDS-PAGE according to Schägger (2006). Runs were performed using 160, 100 and 40 g/L acrylamide for separating, spacing and stacking gels, respectively. The following buffer solutions were used: 1 mol/L Tris–HCl, 1 g/L SDS, pH = 8.45 (gel buffer); 0.1 mol/L Tris–HCl, pH = 8.9 (anode buffer); 0.1 mol/L Tris, 0.1 mol/L tricine, 1 g/L SDS, pH = 8.25 (cathode buffer). Samples were treated with a 0.375 mol/L Tris–HCl (pH = 7), 75 g/L glycerol, 30 g/L SDS, 0.125 g/L Coomasie blue G 250 (Serva). A similar protein mass (20 μg) was loaded onto each lane. Runs were carried out in a Mini Protean II Dual Slab Cell (BIO-RAD). Gels were fixed and stained with Coomasie Brilliant Blue R-250 (1 g/L). Silver staining was applied to increase analytical sensitivity.

2.7. Gel filtration chromatography

Soluble fractions in 35 mmol/L phosphate buffer, pH = 7.8 were analyzed in a Pharmacia LKB, FPLC System (Uppsala, Sweden), using two different molecular exclusion columns. Superdex75 10/300 GL column (exclusion limit: 1×10^5, separation range: 3–70 kDa) (GE Healthcare) was calibrated with blue dextran (exclusion volume, Vo = 7.3 mL), albumin (67 kDa), ovoalbumin (9.2 kDa), chymotrypsin (25 kDa), ribonuclease (19 kDa) and aprotinin (6.5 kDa), obtaining the following calibration equation:

$$\log MM = 1.96 - 2.30\,Kav,$$

where: Kav = (Ve − Vo)/(Vt − Vo), Ve is the elution volume of the resolved species, Vo is the void volume, and Vt is the total volume of the column (Vt = 24 mL). Superdex 30 column (fractionation range $\leq 1 \times 10^4$, total volume = 28 mL) was calibrated with blue dextran (Vo = 8.15 mL), aprotinin (6.5 kDa), a mixture of standard peptides (1046.2, 573.7, 555.6, 379.5 and 238.2 Da) (Sigma) and tyrosine (181.2 Da), appearing all the molecules with molecular mass between 573.7 and 181.2 Da at similar elution volumes (21.3 mL). For both columns, 200 μL of soluble samples were loaded and eluted with 35 mmol/L phosphate buffer, pH = 7.8 at 0.4 mL/min. Polypeptides and peptides were detected by absorbance at 280 nm.

2.8. Antioxidant activity

2.8.1. Scavenging of $ABTS^{+}\cdot$ radical

ABTS radical cation ($ABTS^{+}\cdot$) decoloration assay was performed according to Siddhuraju (2006) with some modifications. ABTS was dissolved in water to a 7 mmol/L concentration. $ABTS^{+}\cdot$ was produced by reaction of ABTS solution with 2.45 mmol/L 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 in a Beckman DU 650 spectrophotometer. Ten μL of sample 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 Trolox (0.5–1.5 mmol/L) was used as positive control (PC). Scavenging % was calculated as follows:

$$\text{Scavenging}\% = [(Abc_0 - Abs_t) - (Abc_0 - Abc_t)/\,Abc_0] \times 100$$

where: Abc_t and Abc_o = absorbance of the negative control at t = 10 min and t = 0, respectively; Abs_t and Abs_o = absorbance of the sample at t = 10 min and t = 0, respectively. For each sample, the undiluted soluble fraction and its 1:2.5, 1:5 and 1:10 dilutions were evaluated by triplicate. Scavenging % was plotted as a function of the protein content of the sample in order to obtain the mass of protein necessary to reduce absorbance by 50% (IC_{50}).

2.8.2. Oxygen radical absorbance capacity (ORAC)

ORAC was determined by a procedure modified from that of Kim, Jang, and Kim (2007). A stock 53.3 μmol/L fluorescein solution in 35 mmol/L phosphate (pH = 7.8) buffer was prepared and maintained in dark at 4 °C. This solution was diluted to 53.3 nmol/L, and a 160 mmol/L AAPH solution in the same buffer was prepared at the moment of the assay. Fluorescein solution (1500 μL) was mixed with 25 μL of sample, phosphate buffer (negative control NC) or Trolox, and preincubated at 37 °C for 3 min with agitation at 300 rpm (Termomixer Eppendorf). AAPH (250 μL) was added and the reaction mixture was incubated at 37 °C. Fluorescence intensity (λ_{exc}: 485 nm, λ_{em}: 535 nm) was monitored for 25 min (Twinkle LBQ70 spectrofluoremeter, Berthold Technologies). For each sample, 1:50, 1:100, 1:250 and 1:500 dilutions of soluble fraction were evaluated in duplicate. Differences of areas (net area) under the fluorescein decay curves between the blank (without AAPH) and each sample were calculated. A standard curve was obtained by plotting the net area as a function of the Trolox concentration (6–100 μmol/L), and the Trolox equivalent value (TE) was obtained for each sample. TE values were plotted versus the protein concentration in samples. ORAC value was expressed as the Trolox concentration equivalent to 100 μg of proteins.

Table 1
Effect of different simulated gastrointestinal digestion conditions on HD and solubility of amaranth proteins.

Sample	Pe/Prot (g/g)	t_1 (min)	Pa/Prot (g/g)	t_2 (min)	HD %	MLP	Protein conc. soluble fraction (mg/mL)
I	–	–	–	–	–		6.23 ± 0.23
Ic	–	60	–	60	0.7 ± 0.1	143	2.90 ± 0.06
Ipepa1	1/20	60	1/100	60	13.1 ± 1.4	7.6	6.56 ± 0.19
Ipepa2	1/20	60	1/100	120	18.1 ± 1.5	5.5	5.81 ± 0.98
Ipepa3	1/10	60	1/10	30	36.9 ± 1.0	2.7	5.55 ± 0.23
Ipepa4	1/10	60	1/10	60	36.9 ± 0.5	2.7	5.55 ± 0.15
Ipepa5	1/10	90	1/10	30	36.2 ± 1.6	2.8	5.68 ± 0.36
Ipepa6	1/10	90	1/10	60	35.8 ± 0.2	2.8	5.46 ± 0.31

I : protein isolate, *Ic* : protein isolate control, *Ipepa*: gastrointestinal digest of isolate. Results are shown as the mean ± SD of at least two determinations. Pe: pepsin, Pa: pancreatin, Prot: protein, t_1: reaction time with pepsin, t_2: reaction time with pancreatin. MLP : Mean length of the peptide chains. MLP = 100/HD%.

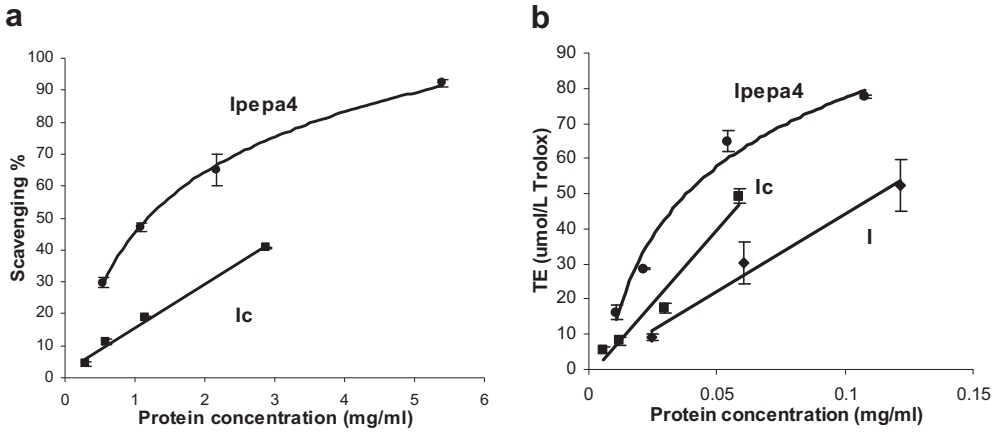

Fig. 1. a) Dose–response curves for $ABTS^{+\cdot}$ scavenging capacity of soluble fractions from: ◆ protein isolate control (*Ic*) sample, ■ digested isolate (*Ipepa4*) sample; $n = 3$. b) Dose–response curves by the ORAC assay of soluble fractions from: ◆ protein isolate (*I*) sample, ■ protein isolate control (*Ic*) sample; and • digested isolate (*Ipepa4*) sample, $n = 2$.

2.9. Statistical analysis

Data were analyzed by means of the analysis of variance (ANOVA) according to the General Linear Model Procedure. When differences were significant ($p < 0.05$) mean values were evaluated by Least Significant Differences (LSD) by the Fisher test using an SYSTAT statistical package (Wilkinson, 1990).

3. Results and discussion

3.1. Simulated gastrointestinal digestion conditions

As mentioned previously, the aim of this work was to release active peptides from amaranth proteins by simulating *in vitro* the gastrointestinal digestion process. Thus, amaranth proteins were first subjected to the action of pepsin (pH = 2) and then to the action of pancreatin (pH = 6). Previous works have demonstrated that antioxidant protein compounds are small peptides, between 2 and 20 amino acids (Chen et al., 1996; Peña-Ramos, Xiong, & Arteaga, 2004). Therefore, hydrolysis conditions were optimized to obtain an extensive hydrolysate containing small peptides. An amaranth protein isolate was used to assay different digestion conditions.

The effect of the reaction conditions on the hydrolysis degree (HD) was evaluated. Results obtained are shown in Table 1. *Ic* sample corresponds to a control system (without enzymes, but using the corresponding buffers). Similar results were obtained for control systems subjected to different reaction times. As shown in Table 1, an increase in pepsin/protein and pancreatin/protein ratios produced a significant increase in the HD ($p < 0.05$). However, an increase in reaction time (from 60 to 90 min for pepsin, and from 30 to 60 min for pancreatin) did not significantly change this parameter ($p > 0.05$). The protein content of freeze-dried products was determined by the microKjeldhal method showing a mean content of 72.4 ± 3.4 g/100 g (w.b.) for control systems and 78.7 ± 6.8 g/100 g (w.b.) for hydrolyzed systems.

Since antioxidant activity assays evaluate soluble components, soluble fractions from freeze-dried samples were prepared using conditions that had been previously optimized in our laboratory to obtain the best protein solubilization. Protein concentration in the soluble fractions is shown in Table 1. It can be observed that the treatment of the protein isolate (*I*) at pH 2 and then at pH 6 to obtain the control system (*Ic*) caused a significant reduction ($p < 0.05$) of soluble protein. According to results previously obtained in our laboratory (unpublished data), a low solubility of amaranth isolates at low ionic strength is located in the range of pH 4–6.3. This decrease is due to the isoelectric point, which is between pH 4.5 and 6.0 (Marcone & Yada, 1992), the isolate proteins precipitate by aggregation induced by the decrease in electrostatic repulsion. In contrast, treatment with pepsin and pancreatin resulted in a significant increase ($p < 0.05$) of soluble protein content as compared to the control system for all the reaction conditions assayed.

To select the best hydrolysis conditions, the antioxidant activity of each hydrolysate was determined by two methodologies: scavenging of the $ABTS^{+\cdot}$ radical and ORAC assays.

3.1.1. $ABTS^{+\cdot}$ scavenging assay

This method measures the discolouration of the $ABTS^{+\cdot}$ radical due to the presence of antioxidant compounds (Re et al., 1999). The procedure used has been optimized in a previous work (Tironi & Añón, 2010). Scavenging % was calculated from measured values, which were plotted as a function of the protein concentration. A dose-dependent activity was observed in all cases. Best adjustment curves were logarithmic in most cases (e.g. for *Ipepa4* sample the corresponding equation was: $y = 27.121\ \mathrm{Ln}(x) + 45.858$, $R^2 = 0.9894$) except for the *Ic* sample ($y = 13.644x + 2.1293$, $R^2 = 0.9907$) (Fig. 1a). The IC_{50} values were calculated using the regression parameters, and are shown in Table 2 in comparison to

Table 2
Antioxidant activity of amaranth protein digests: IC_{50} values for $ABTS^{+}$ assay and ORAC value.

Sample	IC_{50} (mg prot/mL)	ORAC value (μg Trolox/μg prot)
I	10.2 ± 0.8^{a}	0.112 ± 0.018^{a}
Ic	2.98 ± 0.35^{b}	0.199 ± 0.006^{b}
Ipepa1	1.71 ± 0.45^{c}	0.201 ± 0.001^{b}
Ipepa2	$1.65 \pm 0.04^{c,d}$	0.308 ± 0.007^{c}
Ipepa3	1.16 ± 0.09^{d}	0.207 ± 0.001^{b}
Ipepa4	$1.36 \pm 0.26^{d,c}$	0.288 ± 0.003^{c}
Ipepa5	1.17 ± 0.03^{d}	0.276 ± 0.002^{c}
Ipepa6	$1.22 \pm 0.21^{d,c}$	0.199 ± 0.003^{b}
Trolox	0.28 ± 0.04	–

Results in column 2 are shown as the mean ± SD of two independent experiments performed according to Materials and Methods section.
Results in column 3 are shown as the mean ± SD of duplicate measures according to Materials and Methods section.
Different letters (a,b,) in the column indicate significant differences ($p < 0.05$).

those obtained for Trolox, a known antioxidant used as positive control. The value obtained for the *I* sample was comparable to those previously informed by Tironi and Añón (2010) for isolates from *A. mantegazzianus* grown in the years 2006 and 2007. Non-enzymatic treatment (pH = 2 and pH = 6) produced a significant reduction of IC_{50} ($p < 0.05$), indicating an increase in the antioxidant potency of the soluble components. This fact could be related to the insolubilization due to pH treatment of polypeptides with low or no antioxidant activity present in the isolate. In all cases treatment with pepsin and pancreatin led to a reduction of the IC_{50} value respect to the control ($p < 0.05$). Hydrolysates presenting the highest HD (*Ipepa3* to *Ipepa6*) showed a statistically similar activity to *Ipepa2*, which exhibited an approximately 50% lower HD value. In a previous study, a buckwheat protein isolate subjected to pepsin (1/25 g/g protein basis) and pancreatin (1/25 g/g protein basis) hydrolysis exhibited an increase in its $ABTS^{+\cdot}$ scavenging capacity with pepsin (60 min) and pancreatin (120 min) digestion time (Ma et al., 2010). It is important to remark that, although IC_{50} values for hydrolysates were much higher than that for Trolox, the former are a mixture of species with different antioxidant potency, which may also include prooxidant molecules, suggesting the existence of some species with high antioxidant activity.

3.1.2. ORAC assay

In this method, which permits to evaluate the scavenging capacity due to a hydrogen-atom transfer mechanism, the sample is exposed to a peroxyl radical generator (AAPH) and the oxidative degradation of fluorescein is measured (Ou, Hampsch-Woodill, & Prior, 2001). The protective effect of the antioxidants is measured as the area under the fluorescein decay curve (AUC), which permits to evaluate the scavenging degree as well as the radical inhibition time (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002). Different concentrations of each sample and Trolox were assayed, obtaining the corresponding fluorescein decay curves. From the

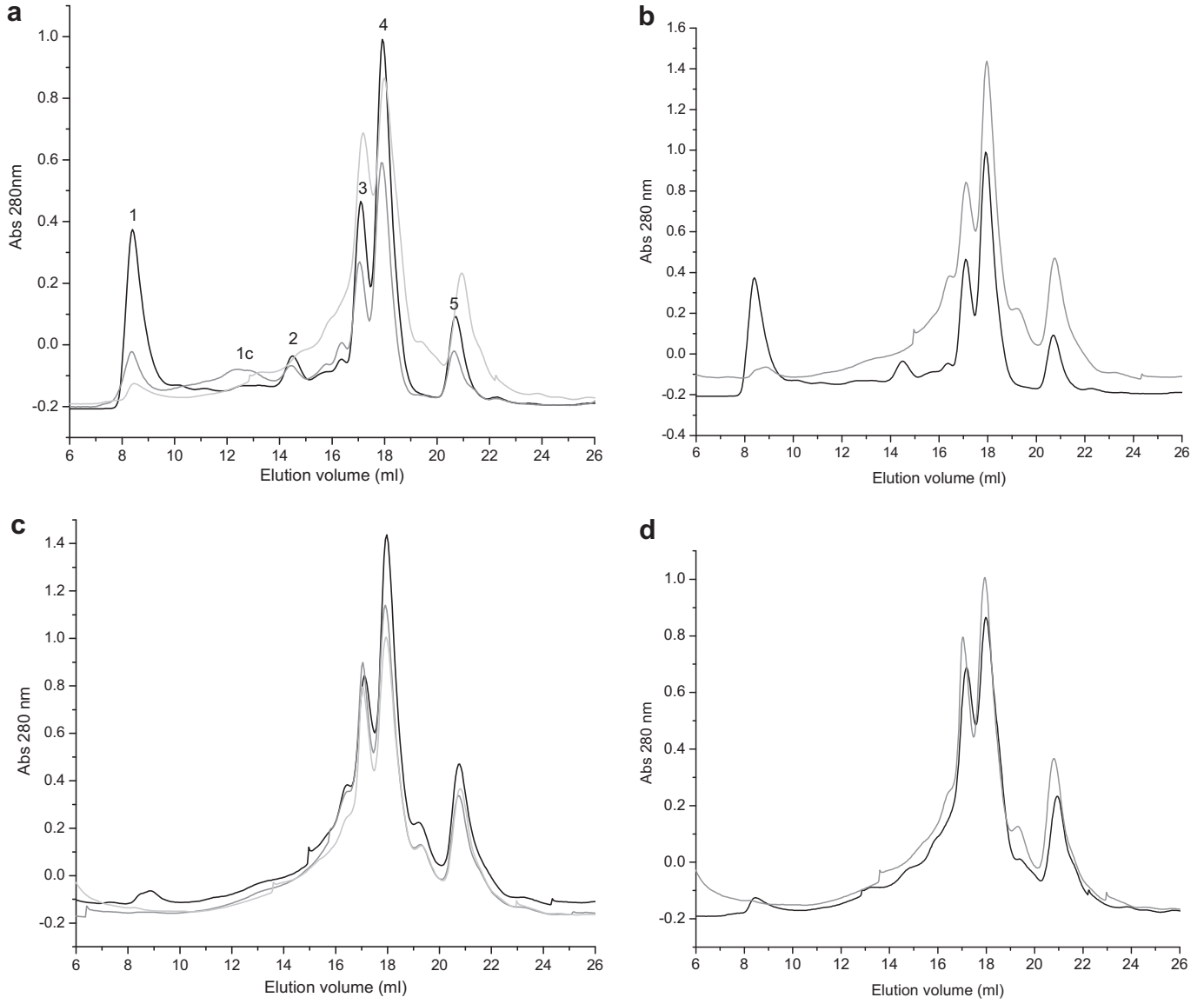

Fig. 2. Gel filtration chromatograms of soluble fractions of different samples in Superdex 75 column: a) — protein isolate *I*, — protein isolate control *Ic*, — digested isolate *Ipepa4*; b) — protein isolate *I*, — alcalase-hydrolysate *H;* c) — alcalase-hydrolysate *H*, — alcalase-hydrolysate control *Hc*, — digested alcalase-hydrolysate *Hpepa*; d) — digested isolate *Ipepa4*, — digested alcalase-hydrolysate *Hpepa*.

AUC values for Trolox, a calibration curve was obtained (AUC = 148.5 $C_{Trolox}(\mu M)$ − 147.29; R^2 = 0.9827), and Trolox equivalent (TE) values were calculated from this curve using the AUC values for each sample assayed. Fig. 1b shows some of the curves obtained plotting TE values as a function of the protein concentration in the sample. While some samples, such as *I* ($y = 438.03x + 0.4602$, $R^2 = 0.9385$), *Ic* ($y = 835.8x - 2.0127$, $R^2 = 0.9667$), *Ipepa3* ($y = 613.4x + 10.633$, $R^2 = 0.9867$) and *Ipepa6* ($y = 917.35\ x - 6.1424$, $R^2 = 0.9932$), presented a better adjustment to a linear dose-response, a second group of samples, including *Ipepa1* ($y = 23.809\ \mathrm{Ln}(x) + 111.41$, $R^2 = 0.9645$), *Ipepa2* ($y = 25.802\ \mathrm{Ln}(x) + 138.91$, $R^2 = 0.9624$), *Ipepa4* ($y = 28.991\ \mathrm{Ln}(x) + 144.34$, $R^2 = 0.9761$) and *Ipepa5* ($y = 28.047\ \mathrm{Ln}(x) + 139.52$, $R^2 = 0.9861$), adjusted better to a logarithmic curve, indicating a radical saturation in the concentration range assayed. These results suggest that the second group of samples would be more active since they would achieve the radical saturation with lower protein concentrations. Since a higher TE value indicates a higher antioxidant capacity, the comparison of the curves allows to appreciate that TE values increased after treatment of the isolate at pH 2 and 6 (Fig. 1b), in concordance with results obtained by the $ABTS^{+}\cdot$ assay. TE values for all digested samples are increased with respect to the corresponding control system, as can be seen in the case of *Ipepa4* in the Fig. 1b. In this case, a linear dose-response could be observed up to a protein concentration of about 0.06 mg/mL. With the aim to compare the antioxidant potency of the hydrolysates, the ORAC value was expressed as μg Trolox/μg protein. As shown in Table 2, *Ipepa2*, *Ipepa4* and *Ipepa5* samples displayed the highest antioxidant activity as measured by this methodology. This assay showed again that simulated gastrointestinal digestion released antioxidant peptides. However, the antioxidant potencies of *Ipepa3* and *Ipepa6* systems were similar ($p > 0.05$) to the potency of the control system (*Ic*). According to these results, ORAC values did not depend on the hydrolysis degree in the range assayed since samples *Ipepa3* to *Ipepa6* presented similar HD but different ORAC behaviour. In addition, *Ipepa2* showed a similar ORAC value to *Ipepa4* and *Ipepa5*, but a much lower HD. Although $ABTS^{+}\cdot$ scavenging behaviour was not exactly equivalent to that previously described for ORAC, this

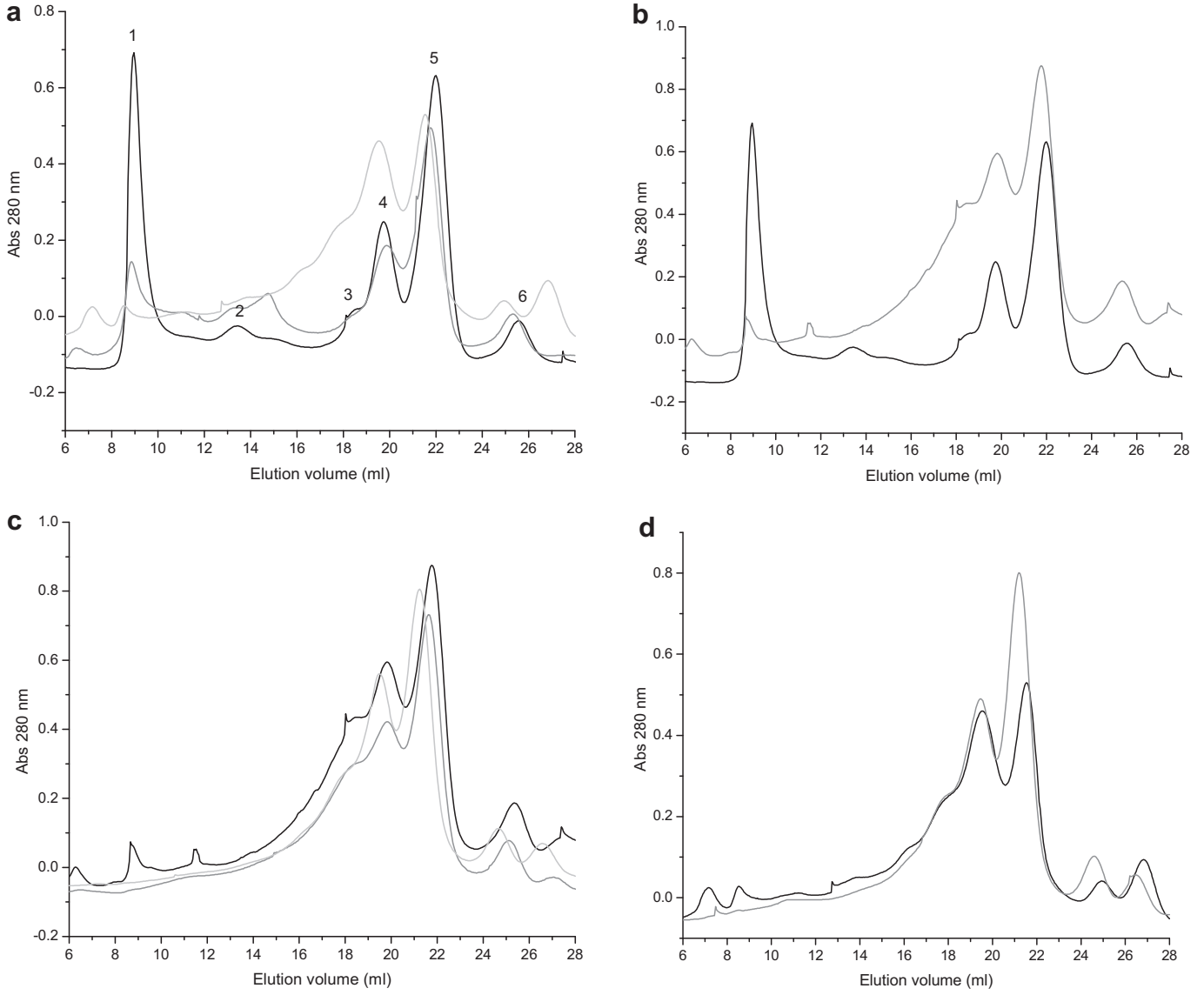

Fig. 3. Gel filtration chromatograms of soluble fractions of different samples in Superdex 30 column: a) — protein isolate *I*, — protein isolate control *Ic*, — digested isolate *Ipepa4*; b) — protein isolate *I*, — alcalase-hydrolysate *H*; c) — alcalase-hydrolysate *H*, — alcalase-hydrolysate control *Hc*, — digested alcalase-hydrolysate *Hpepa*; d) — digested isolate *Ipepa4*, — digested alcalase-hydrolysate *Hpepa*.

activity was neither dependent on the HD. These results suggest that the nature of peptides, rather than their size, would be an important factor determining the scavenging activity. Clausen, Skibsted, and Stagsted (2009) studied the scavenging activity of milk proteins as well as of free amino acids by the $ABTS^{+}\cdot$ and ORAC assays, concluding that both composition and solvent exposure of amino acids would play crucial roles for the activity of proteins. In contrast, free amino acids presented higher potencies by ORAC than by $ABTS^{+}\cdot$ assay, which could be related to the bigger size of $ABTS^{+}\cdot$ compared with the peroxyl radicals (Clausen et al., 2009). In addition, the $ABTS^{+}\cdot$ method measures exclusively the ability of antioxidants to act as hydrogen or electron donor to neutralize preformed radicals, while ORAC measures both the antioxidant capacity to inhibit the radical initiation as well as the neutralization of the formed radicals (Ma et al., 2010). These facts could explain the differences between both methodologies.

The ORAC assay reproduces physiological conditions because peroxyl radicals are present in the human organism. These radicals are formed by homolysis of hydroperoxydes formed during fatty acid oxidation (Almeida, Fernández, Lima, Costa, & Bahia, 2008). According to previous results, we decided to select for further studies the digestion conditions applied to *Ipepa4*, because this sample presented good antioxidant capacity by both methods assayed, as well as a high hydrolysis degree. Mean length of the peptide chains (MLP) can be estimated from HD according to MLP = 100/HD % (Adler-Nissen, 1986). In this way, *Ipepa2* (HD = 18.1%) presented an MLP value of 5.5 amino acids, while *Ipepa4* (HD = 36.9%) showed an MLP value of 2.7 amino acids (Table 1). Thus, the last sample could better reflect the *in vivo* gastrointestinal digestion process.

3.2. Simulated gastrointestinal digestion of A. mantegazzianus isolate after alcalase hydrolysis

Selected digestion conditions were applied to an amaranth alcalase-hydrolysate in order to compare the results with those obtained for the non-hydrolyzed isolate, and to evaluate these samples as potential functional ingredients. The alcalase-hydrolysate (*H*) used presented an HD = 29.2 ± 1.3%. This kind of amaranth hydrolysate has demonstrated antioxidant activity by the $ABTS^{+}\cdot$ radical scavenging assay as well as by the linoleic acid oxidation inhibition assay (Tironi & Añón, 2010).

3.2.1. Hydrolysis degree

We evaluated the progression of HD after each simulated gastrointestinal digestion step for the isolate and the alcalase-hydrolysate. Although initial HD values were very different, 1.4 ± 0.7 and 29.2 ± 1.3 for *I* and *H* respectively and after treatment with pepsin was observed an increase in HD values in both cases, (7.4 ± 0.4 and 34.1 ± 1.9), the finals values, after treatment with pancreatin, not presented significant differences, 36.9 ± 0.5 for *I* and 42.0 ± 2.6 for *H*. According to these results, the action of gastrointestinal enzymes was reduced when a previous hydrolysis with alcalase was performed. In particular, pancreatin showed a very limited activity on this substrate. A possible explanation for this could be related to the fact that alcalase, similar to various digestive proteases, is a serine protease, but with a wide range of specificity (Chabanon, Chevalot, Framboisier, Chenu, & Marc, 2007). Thus, alcalase could cleave some sites that constitute specific targets for pancreatin action.

3.2.2. Molecular characterization of samples

3.2.2.1. Gel filtration chromatography. As was previously mentioned in Materials and methods section, molecular characterization of soluble samples was performed by size exclusion chromatography using two columns with different range of resolution. Fig. 2 shows the chromatograms corresponding to Superdex 75 column (resolution range: 3–70 kDa). In the case of the isolate, its soluble fraction I presented five main peaks (Fig. 2a). After treatment of the isolate at pH = 2 and then at pH = 6, the soluble fraction *Ic* showed considerable changes in the chromatogram such as an important decrease in the area of peak 1 (molecules between 40 and 70 kDa) as well as a not so important decrease in peaks 3, 4 and 5 (molecules lower than 6.5 kDa). The diminution of these species would be responsible for the lower solubility of *Ic* sample. In addition, a new peak (1c – Fig. 2a) with a molecular mass between 19 and 25 kDa appeared. After digestion (*Ipepa4* sample), an additional decrease of peaks 1 and 1c was evidenced indicating that these polypeptides were attacked by the enzymes, yielding diverse molecules with molecular weight lower than 19 kDa (Fig. 2a). The soluble fraction obtained from the isolate previously hydrolyzed with alcalase (H) also presented an important diminution of peak 1 accompanied by the appearance of diverse molecules with a molecular mass lower than 25 kDa (Fig. 2b). When the alcalase-hydrolysate was treated at pH = 2 and pH = 6, the soluble fraction Hc showed some changes as compared to the untreated hydrolysate, including the complete disappearance of peak 1 and a reduction of the other peaks (Fig. 2c). Treatment with pepsin and pancreatin did not produce important additional changes in the chromatographic profile (*Hpepa*), except for a small increase of molecular species at an elution volume of about 16 mL and also in peak 4 (Fig. 2c). Thus, a comparison between digested samples (*Ipepa4* and *Hpepa*), reveals that *Hpepa* contained much more molecules with molecular weigh between 19 and 3 kDa (Fig. 2d).

To analyze the molecules of low molecular weight, the same samples were run in a Superdex 30 column (exclusion size < 10 kDa). *I* samples presented six main peaks (Fig. 3a). After treatment at pH = 2 and pH = 6 (*Ic* sample) a reduction of peak 1 (molecules with mass larger than 10 kDa) and peak 5 (molecular weight lower than 1 kDa) was observed, concomitant with the appearance of molecules of about 10 to 6 kDa, (Fig. 3a). Digestion with pepsin and pancreatin (*Ipepa4*) produced the loss of molecules bigger than 10 kDa (peak 1) and a considerable increase in the abundance of diverse molecules lower than 6.5 kDa, including very small molecules (peaks 6 and 7) (Fig. 3a). Soluble fraction from alcalase-hydrolysate (*H*) contained a very high proportion of

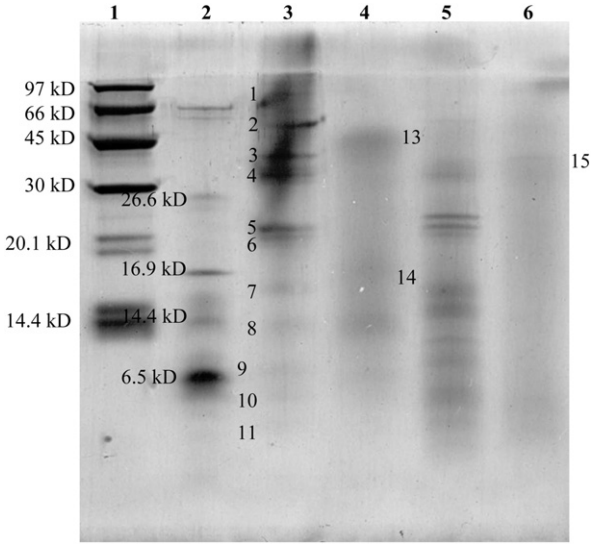

Fig. 4. Tricine-SDS-PAGE protein profiles of soluble fractions: 1) low molecular weight standards; 2) very low molecular weight standards; 3) protein isolate *I*; 4) digested isolate *Ipepa4*; 5) alcalase-hydrolysate *H.*; 6) digested alcalase-hydrolysate *Hpepa*.

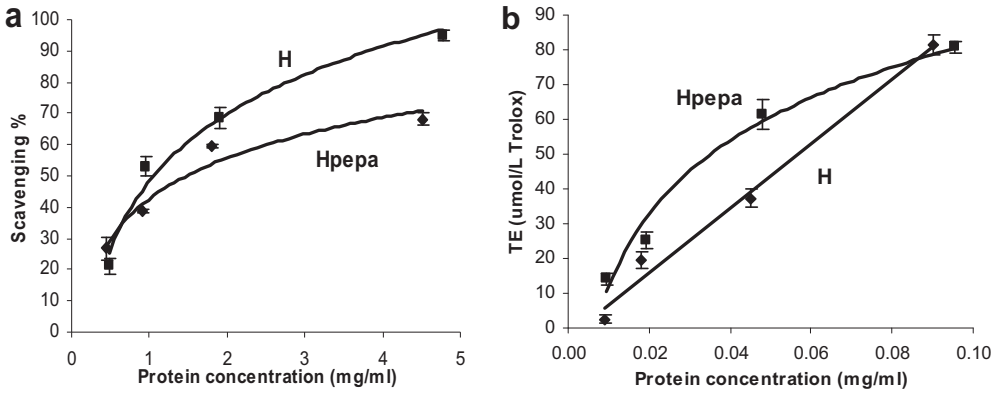

Fig. 5. Antioxidant activity of alcalase-hydrolysate *H* (◆) and its gastrointestinal digest *Hpepa* (■): a) $ABTS^{+}$ scavenging, b) ORAC assay.

molecules with mass lower than 6.5 kDa (Fig. 3b). After pH treatment of this sample, all peaks decreased. Molecules of about 1 kDa and lower appeared after treatment with pepsin and pancreatin (*Hpepa*) (Fig. 3c). Comparison of *Ipepa4* and *Hpepa* samples showed a higher proportion of molecules lower than 1 kDa in the latter (Fig. 3d).

3.2.2.2. Tricine-SDS-PAGE. As another way to molecular characterization of soluble samples, tricine-SDS-PAGE was carried out in order to evaluate mainly small peptides. It was possible to detect molecules with molecular weights around 5 kDa or higher as suggested by the fact that standards of 3.50 and 1.42 kDa could not be detected. Protein profiles are shown in Fig. 4. The non-hydrolyzed isolate sample I (Fig. 4 – lane 3) contained molecules with molecular weights above 26.6 kDa that appeared in the upper portion of the gel, which could correspond to the acid subunits of the amaranth 11S globulin (29.3 and 32.0 kDa). Also, a band probably related to the basic subunits of these proteins (20.8 kDa), as well as diverse bands lower than 20 kDa corresponding to albumins can be observed (Segura-Nieto et al., 1994). After the hydrolysis of the isolate with pepsin and pancreatin, all bands corresponding to molecular mass higher than 14 kDa disappeared, appearing two new weak bands (13 and 14) (Fig. 4 – lane 4). However, the diminution of the total intensity of the *Ipepa4* sample in the gel suggests that most peptides generated by gastrointestinal digestion simulation had a molecular weight lower than the detection limit of this gel. Some intense bands observed in the upper portion of the gel (>30 kDa) in the non-hydrolyzed isolate (Fig. 4 – lane 3) disappeared after alcalase-hydrolysis (Fig. 4 – lane 5), while new bands appeared after this treatment, evidencing the generation of peptides of diverse molecular weight, specially lower than 16.95 kDa. After hydrolysis of the alcalase-hydrolysate with pepsin and pancreatin (Fig. 4 – lane 6), a diminished intensity was observed in all bands present in H sample, indicating that these polypeptides were attacked by the digestive enzymes and that small peptides, not detected on this gel were released, as is suggested by the diminution in the total intensity. Only a new small band with molecular weight higher than 30 kDa (band 15 – Fig. 5, lane 6) could be observed as a product of the digestion of H. After simulated gastrointestinal digestion, the electrophoretic profiles of isolate and alcalase-hydrolysate presented some differences, evidencing the presence of different molecular species in each sample as was observed by gel filtration chromatography.

3.2.3. Antioxidant activity

3.2.3.1. $ABTS^{+}$·scavenging. Fig. 5a shows the dose–response plots of the scavenging activity of the alcalase-hydrolysate both before and after pepsin and pancreatin digestion (*H* and *Hpepa*, respectively). A logarithmic behaviour was observed in both cases ($y = 18.197\ \mathrm{Ln}(x) + 43.586$, $R^2 = 0.9364$, for *H* and $y = 30.99\ \mathrm{Ln}(x) + 48.377$, $R^2 = 0.9761$ for *Hpepa*). IC_{50} values calculated from regression parameters were 1.44 ± 0.03 mg/mL for *H* and 1.02 ± 0.04 mg/mL for *Hpepa*. Alcalase-hydrolysis increased the antioxidant potency, as shown by the fact that the IC_{50} value for *H* was significantly lower than that for for *I* ($p < 0.05$), in agreement with previous reports (Tironi & Añón, 2010). However, after the simulated gastrointestinal digestion the antioxidant activity of *Hpepa* did not differ significantly from that of *H* ($p > 0.05$). Comparing *Ipepa4* and *Hpepa* samples, a previous hydrolysis with alcalase did not improve the antioxidant potency of the amaranth protein isolate as evaluated by the present methodology, being the corresponding IC_{50} values statistically similar ($p > 0.05$).

3.2.3.2. ORAC assay. Fig. 5b presents the dose–response curves for *H* and *Hpepa* regarding their absorbance capacity of peroxyl radicals, showing *H* sample a linear behaviour ($y = 923.51x - 2.3966$, $R^2 = 0.9848$) and *Hpepa*a logarithmic behaviour in the concentration range assayed ($y = 30.414\ \mathrm{Ln}(x) + 151.81$, $R^2 = 0.9755$). In the last case, a linear response was observed up to a protein concentration of 0.05 mg/mL. Results showed that simulated gastrointestinal digestion would produce an increase in the antioxidant activity of alcalase-hydrolysate, especially at lower protein concentrations. ORAC values obtained were 0.22 ± 0.01 and 0.30 ± 0.01 μg Trolox/μg protein for *H* and *Hpepa*, respectively. As in the case of the $ABTS^{+}$·assay, the antioxidant potency of *Hpepa* was similar to that of *Ipepa4* ($p > 0.05$). The results obtained show amaranth hydrolysates as products with a good antioxidant potency as compared to other hydrolysates found in the literature. For example, caseinate hydrolysed with alcalase presented a TE value of about 4.5 μmol/L Trolox/50 μg proteins (Kim et al., 2007), while in our case values were between 40 and 60 μmol/L Trolox/50 μg proteins for all digests. Hernández-Ledesma et al. (2005) showed that β-lactoglobulin and α-lactalbumin hydrolysed with diverse enzymes yielded TE values between 1 and 2.9 μmol Trolox/mg proteins in the first case and between 0.7 and 2.1 μmol Trolox/mg proteins in the second case, being these values comparable to those obtained in the present study (between 0.8 and 1.2 μmol Trolox/mg proteins for the different digests).

5. Conclusions

The results obtained in the present study show amaranth (*A. mantegazzianus*) proteins as a potential source of antioxidant peptides which could be released into the human organism after

gastrointestinal digestion. A previous hydrolysis of the amaranth protein isolate with alcalase did not improve the antioxidant activity obtained by simulated gastrointestinal digestion. In addition, the digestion process did not diminish the antioxidant capacity of the alcalase-hydrolysate. In this way, both the amaranth protein isolate and the alcalase-hydrolysate showed a potential capacity to scavenge free radicals after gastrointestinal digestion. This study constitutes a first approach to the evaluation of amaranth proteins as ingredients to formulate functional foods with antioxidant activity. Further work is been performed in our laboratory to elucidate the real relevance of the generated antioxidant peptides in the human body.

Acknowledgements

This work was supported by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, Argentina) projects (PICT 2007-1114 and PICT 2007-1119). Authors Tironi and Añón are members of CONICET (Argentina).

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