Amaranth Peptides from Simulated Gastrointestinal Digestion: Antioxidant Activity Against Reactive Species

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Abstract We evaluated the capacity of simulated gastrointestinal digests or alcalase hydrolysates of protein isolates from amaranth to scavenge diverse physiologically relevant reactive species. The more active hydrolysate was obtained with the former method. Moreover, a prior alcalase treatment of the isolate followed by the same simulated gastrointestinal digestion did not improve the antioxidant capacity in any of the assays performed and even produced a negative effect under some conditions. Gastrointestinal digestion produced a strong increment in the scavenging capacity against peroxyl radicals (ORAC assay), hydroxyl radicals (ESR-OH assay), and peroxynitrites; thus decreasing the IC_{50} values to approximately 20, 25, and 20 %, respectively, of the levels attained with the nonhydrolyzed proteins. Metal chelation (HORAC assay) also enhanced respect to isolate levels, but to a lesser extent (decreasing IC_{50} values to only 50 %). The nitric-oxide– and superoxide-scavenging capacities of the digests were not relevant with respect to the methodologies used. The gastrointestinal digests from amaranth proteins acted against reactive species by different mechanisms, thus indicating the protein isolate to be a potential polyfunctional antioxidant ingredient.

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Introduction

Although oxygen is essential for life, the presence of that vital gas is also associated with the generation—even under physiologic conditions—of reactive oxygen and nitrogen species, which are responsible for oxidative damage to biologic macromolecules [1] and involved in a variety of pathologic situations (*e.g.*, mutation, carcinogenesis, degenerative and cardiovascular diseases, inflammation, aging) [2]. Some of the reactive species are radicals such as the peroxyl (ROO·), hydroxyl (OH·), superoxide anion ($O_2 \cdot^-$), nitric oxide (·NO), and singlet oxygen ($^1O_2$) species; while others are nonradical reactive molecules such as hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), and the peroxynitrite anion (ONOO·⁻). The human organism has diverse complementary antioxidant mechanisms that act on different oxidants and/or specific cell compartments: some of these activities are enzymatic; while others operate *via* low-molecular-weight molecules that have the ability to scavenge free radicals, thus inhibiting the initiation and/or propagation of the radical chain reactions mainly through the donation of a |hydrogen atom. Other antioxidant activities occur through the chelation of the transition metals that are responsible for the generation of OH· radicals by the Fenton reaction [3].

Diverse peptides, released from animal or plant proteins by *in-vitro* or *in-vivo* processes, have been reported to show beneficial health effects, including antioxidant activity [4]. This antioxidant capability of those peptides is related to the associated protease activity; the extent of the hydrolysis; and the peptides' structural characteristics such as molecular size, hydrophobicity, and amino-acid composition [5, 6]. Since the

mechanisms of action of these peptides have not been fully characterized; much effort is currently being exerted through the use of the different *in-vitro* methods that are appropriated for the study of each action.

Amaranth is an ancestral American pseudocereal plant. Different substances present in the amaranth seeds exhibit diverse biologic activities [7]. The results from a previous study on *Amaranthus mantegazzianus* seeds demonstrated the presence of naturally occurring peptides and polypeptides that scavenged free radicals and inhibited the oxidation of linoleic acid. Hydrolysis by alcalase (aka subtilisin, a nonspecific protease originally obtained from *Bacillus subtilis*) improved that scavenging activity [8]. Therefore, in order to evaluate the potential release of antioxidant peptides in the body by gastrointestinal digestion, we subjected amaranth-protein isolates and their alcalase hydrolysates to a form of simulated gastrointestinal digestion and then evaluated the capacity of those digests to scavenge free radicals [9]. Taking into account our interest in studying the potential of amaranth-protein isolates and hydrolysates as ingredients in a formulation of functional antioxidant foods, we decided to extend the earlier work measuring the activity of the gastrointestinal digests *versus* reactive-oxygen and reactive-nitrogen species of physiopathologic relevance in order to evaluate the potential capacity of those isolated peptides in combating oxidative insult. The aim of these present investigations was therefore to analyze the antioxidant capacity of amaranth-derived peptides generated by simulated gastrointestinal digestion against different reactive species in order to evaluate the possible mechanisms of action as well as the effect of a previous alcalase hydrolysis of the amaranth isolate on that scavenging activity.

Materials and Methods

Alcalase 2.4 L (*Bacillus licheniformis*, Novozyme Corp), Trolox, nitro blue tetrazolium (NBT), 2,4,6-trinitrobenzenesulfonic acid (TNBS), bovine-serum albumin, phenazine methosulfate (PMS), diethylenetriaminepentaacetic acid (DTPA), ß-nicotinamide adenine dinucleotide reduced dipotassium salt (NADH), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), ethylenediaminetetraacetic acid tetrasodium salt (EDTA), α-phenyl-N-t-butylnitrone (PBN), 2,7-dihydrodichorofluorescein diacetate, (DCFH) and $FeSO_4.7H_2O$ were purchased from Sigma Chemical Co. (St. Louis, MO, USA); pepsin 1:15,000, 5X NF standards, and porcine Pancreatin 4X at 100 USP units/mg from MP Biomedicals LLC (Solon, OH, USA); sodium fluorescein from Fluka (Steinheim, Germany); and chlorogenic acid and 2,2′-azo-bis-(2-methylpropionamidine) dihydrochloride (AAPH) from Aldrich (WI, USA).

Samples

Protein Isolate (I) From *A. mantegazzianus* (Pass cv Don Juan) -grown at the School of Agronomy, National University of La Pampa (Argentina)- *I* was obtained by the methodology previously used in our laboratory [8, 10]. The isolate's composition (values/100 g) was: 79.1±0.1 g proteins, 9.7±0.4 g carbohydrates; 7.0±0.3 g water, 3.2±0.1 g ash, and 1.7±0.2 g lipids.

Alcalase Hydrolysate (H) *H* was prepared from *I* according to a previously optimized method [8]. The degree of hydrolysis (DH) was measured by the reaction of free amino groups with TNBS through the protocol previously applied to amaranth proteins in our laboratory [8].

Simulated Gastrointestinal Digests (Id and Hd). The simulated digestion process has been previously optimized for amaranth proteins [9]. The method stated in brief: The sample (*I* or *H*) was initially treated with a pepsin solution (30 mM NaCl in 0.1 N HCl, pH 2) at a pepsin/protein ratio of 1/10 (w/w) and 37 °C with agitation, for 60 min. The pH was then adjusted to 6, and a 0.4 % (w/v) pancreatin solution in 0.1 M $NaHCO_3$, pH 6 was added at a pancreatin/protein ratio of 1/10 (w/w), followed by an incubation at 37 °C with agitation for 60 min. After stopping the reaction by heating at 85 °C for 10 min, the suspensions were freeze-dried. The DH was measured as was previously mentioned.

Preparation of Soluble Fractions Suspensions (10 mg/mL) of freeze-dried samples in 35 mM phosphate buffer (pH 7.8) were prepared by agitation at 500 rpm (1 h, 37 °C) in an Eppendorf Thermomixer followed by centrifugation (10,000×*g*, 10 min, room temperature, Spectrafuge 24D, Lab Net International). The soluble-protein concentration was determined by the Lowry method [11].

Antioxidant Activity

Oxygen-Radical-Absorbance Capacity (ORAC) The ORAC assay was based on a previously described procedure [9] adapted here to use with 96-well black-microplates. Of a 53.3 nM fluorescein solution in the phosphate buffer, 150 μl was mixed with 25 μL of sample or the same volume of either the phosphate buffer (negative control) or Trolox (positive control), then preincubated at 37 °C for 10 min. To that mixture, 25 μl of 160 mM, AAPH in the phosphate buffer was added and the reaction mixture incubated at 37 °C for 45 min, at which time the fluorescence intensity (λ_{exc}: 485, λ_{em}: 535 nm) was read every min in a SYNERGY HT–SIAFRT™ multidetection microplate reader (Biotek

Instruments, USA) to obtain the fluorescein-decay curve, the area under curve (AUC) being: AUC = 0.5 + f1/f0 + f2/f0 + …+ fi-1/f0 + 0.5 fi/f0, where f is the fluorescence value at a particular time during the decay. A blank without AAPH was included and the percent scavenging calculated as: % ROO· scavenging = $[(AUC_S–AUC_{NC})/(AUC_B-AUC_{NC})]\times100$; where S = sample, B = blank, NC: negative control. Trolox (6.25–75.0 μM) was used as a reference compound.

Hydroxyl-Radical-Averting Capacity (HORAC) The hydroxyl radical was generated by a cobalt-mediated Fenton-like reaction with fluorescein used as a probe [12, 13]. A 60.3 nM fluorescein solution in the phosphate buffer and an aqueous 0.75 M H_2O_2 solution were prepared. A cobalt solution was constituted by dissolving 10 mg of picolinic acid and 11 mg of $CoCl_2.6H_2O$ in 50 mL of water. Either samples or buffer (20 μL) were mixed with 190 μL of the fluorescein solution, 15 μL of the H_2O_2 solution, and 75 μL of the cobalt solution; incubated at 37 °C for 3 h in the SYNERGY microplate reader; and the fluorescence (λ_{exc}: 485, λ_{em}: 535 nm) read at 1-min intervals to obtain the AUC. The percent inhibition was calculated as: % OH· inhibition = $[(AUC_S–AUC_{NC})/(AUC_B-AUC_{NC})]\times100$; where: S = sample, B = blank (without addition of the cobalt and hydrogen-peroxide solutions), and NC = negative control. Chlorogenic acid (0.05–0.5 mg/mL) was used as a reference compound.

Scavenging of the Superoxide Radical This assay is based on a nonenzymatic PMS/NADH system that generates superoxide radicals, which species reduce NBT to a purple formazan [14]. To 20 μL of sample or buffer (blank) and 80 μL of 30 μM PMS solution, were added 100 μL of 150 μM NADH solution, 100 μL of 100 μM NBT solution. After 2 min of agitation at room temperature, the absorbance at 560 nm was read in the SYNERGY microplate reader. The percent scavenging was calculated as: % O_2· scavenging = $[(A_B–A_S)/A_B]\times100]$; where: A_B = absorbance of the blank, A_S = absorbance of the sample. Ascorbic acid (0.025–1.0 mM) was used as a reference compound.

Scavenging of the Nitric-Oxide Radical Nitric oxide (·NO) was generated by the decomposition of 200 mM SNP in phosphate-buffered saline solution (30.0 mM Na_2HPO_4, 19.0 mM NaH_2PO_4, pH 7.4). Reaction mixtures containing 90 μL of the sample or buffer (blanks) and 10 μL of SNP were incubated at room temperature for 150 min, 100 μL of the Griess reagent were added, and the nitrite concentration remaining was determined by the absorbance at 540 nm (SYNERGY HT reader). The percent scavenging was calculated as: %· NO scavenging = $[(A_B–A_S)/A_B]\times100$; where: A_B = the absorbance of the blank and A_S = the absorbance of the sample. Ascorbic acid (0.5–3.0 mg/ml) was used as a standard [14, 15].

Scavenging of Peroxynitrite The peroxynitrite ion (ONOO·⁻) was synthesized by mixing 50 mM $NaNO_2$ with 50 mM H_2O_2 (final volume, 10 mL) in an ice bath; 5 mL of 1 M HCl solution were added, and the reaction was stopped immediately by the addition of 5 mL of 1.5 M NaOH [16]. In order to eliminate excess H_2O_2, 5 mL of 0.6 M MnO_2 (previously washed in 3 M NaOH solution) was added and the resulting suspension filtered after 30 min. The ONOO·⁻ concentration was determined by measuring the absorbance at 302 nm ($\varepsilon=1$, 670 $M^{-1}.cm^{-1}$). The scavenging assays were performed with DCFH as a fluorescent probe. DCFH was obtained by treatment of 0.5 ml of 1 mM DCFH-diacetate solution in absolute ethanol with 2 ml of 0.1 M NaOH. After 30 min of incubation at room temperature, 7.5 ml of phosphate buffer (50 mM Na_3PO_4, 90 mM NaCl, 5 mM KCl, pH 7.4) supplemented with 100 μM DTPA (included right before the assay) were added to give a final concentration of DCFH of 50 μM [17]. Reaction mixtures containing 50 μL of 20 μM DCFH, 25 μL of 200 nM ONOO·⁻ (in 0.1 N NaOH), and 50 μL of sample were incubated for 5 min at 37 °C. The fluorescence (λ_{exc}: 485 nm, λ_{em}: 535 nm) was then read in the SYNERGY reader [18]. The percent scavenging was calculated as: % ONOO·⁻ scavenging = $[(F_B – F_S)/F_B)\times100]$; where: F_B = fluorescence intensity of the blank, A_S = fluorescence intensity of the sample. Gallic acid (1–250 μg/mL) was used as a standard.

Electron-Spin-Resonance (ESR) Determinations The scavenging of AAPH-derived and OH· radicals was evaluated by ESR at room temperature in a Bruker BioSpin EMX Plus spectrometer (Bruker, Karlsruhe, Germany). The AAPH-derived radicals were trapped with PBN in the following manner: Reaction mixtures containing 40 μL of 50 mM PBN dissolved in the phosphate buffer, 40 μL of sample, and 20 μL of 100 mM AAPH (in the phosphate buffer) were incubated at 37 °C for 30 min; at the end of which time the reaction mixtures were transferred to Pasteur pipettes and immediately measured. Typical spectrometer settings were: field-modulation frequency, 50 kHz; microwave frequency, 9.81 GHz; modulation amplitude, 1 G; microwave power, 20 mW; time constant, 82 ms; sweep width, 50 G; center field, 3515 G; number of scans, 1. OH· were generated by the Fenton reaction and trapped by DMPO. Reaction mixtures containing 45 μL of the sample in the phosphate buffer, 10 μL of 1 M DMPO, 25 μL of 1 mM H_2O_2, 10 μL of 1 mM EDTA, and 10 μL of 1 mM $FeSO_4$ were transferred to Pasteur pipettes and immediately measured. Typical spectrometer settings were as previously indicated except for: modulation amplitude, 0.5 G; time constant, 655 ms; and sweep width, 100 G. The intensity of the signals was quantified by determining the peak-to-trough height of selected spectral lines. The percent

scavenging of radicals was calculated as: Scavenging % = $[(\Delta H_B - \Delta H_S)/\Delta H_B) \times 100]$; where: ΔH_B = peak-to-trough height of the blank and ΔH_S = peak-to-trough height of the sample. Trolox (10.0–250 μM in the phosphate buffer) and chlorogenic acid (0.125–1.00 mg/mL in water) were used as reference compounds for the AAPH-derived and OH· radicals, respectively.

Statistical Analysis

Each assay measurement was performed at least in triplicate. The percent inhibition or scavenging was plotted *versus* the protein concentration of the samples. Curves were fitted by nonlinear regression according to the sigmoideal dose–response (variable slope) equation: Y = bottom + (top-Bottom)/$(1+10^{[\log IC_{50}-X]}$*Hill Slope), where X=log (concentration), Y = % radical inhibition. The data were generated by the GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California, USA. In order to normalize the curves, the variables *bottom* and *top* were constrained to constant values of 0 and 100, respectively. The concentration value that inhibited 50 % of the radicals (IC_{50}) was then obtained and differences between samples evaluated by an F test ($p < 0.01$).

Results and Discussion

The hydrolysates presented the following DH: *H*: 29.2± 1.3 %, *Id*: 36.9±0.5 %, and *Hd*: 42.0±2.6 %.

AAPH-Derived-Radical–Scavenging Capacity AAPH is decomposed by heating to generate three possible species, the alkyl (R·), peroxyl (ROO·), and alkoxyl (RO·) radicals, where R represents the $H_2N(HN)C–C(CH_3)_2$ group [19]. The ORAC assay measures the hydrophilic chain-breaking antioxidant capacity against ROO· radicals induced by AAPH, which reactions proceed according to a classic proton-transfer mechanism [20]. ROO· scavenging (ESM 1a) increased after the hydrolysis of *I* by alcalase (preparation *H*) and/or gastrointestinal enzymes (preparation *Id*). The IC_{50} value, calculated from the sigmoidal dose–response-curve fitting (Table 1), diminished 5-fold after the digestion of *I* with alcalase (*H*) or with simulated gastrointestinal enzymes (*Id*), indicating a strong increment in the potency of these hydrolysates as ROO· scavengers ($p < 0.01$). Simulated gastrointestinal digestion of *H*, however, produced a small decrease ($p < 0.01$) in the *Hd* potency. The ROO· -scavenging capacity of other protein hydrolysates as estimated by the ORAC assay has been previously demonstrated. Casein hydrolysates with alcalase (pH 7) had been reported to exhibit a Trolox-equivalent (TE)

value of about 2 μM/20 μg/mL of hydrolysate [21]; whereas, in the present work the corresponding value was about 20 μM, thus indicating a considerably higher antioxidant potency of *H*, *Id*, and *Hd*. Samaranayaka & Li-Chan [22] reported an ORAC value of 225 μmol TE/g of freeze-dried fish protein hydrolysate; while different hydrolysates obtained from rice-protein isolates had ORAC values between 34.2 and 87.3 μmol TE/g of dry sample [23]. The present values were much higher, no doubt because of the mean protein content of 79 % in the freeze-dried samples [9]. The ORAC values (normalized to the mean protein content) were at about 950 μmol TE/g of freeze-dried sample for *Id* and *H*, and 630 μmol TE/g of freeze-dried sample for *Hd*. As in the present work, after *in-vitro* digestion, the antioxidant capacity assessed by the ORAC assay of a flaxseed-protein isolate was 30 % higher than the value obtained after the corresponding alcalase hydrolysis [24]. The antioxidant capacity of peptides is dependent on size as well as on amino-acid composition, sequence, and structural characteristics [5, 6]; these considerations would explain the notable differences between hydrolysates obtained from different protein sources as well as from different operational conditions (*i.e.*, different proteases or extents of hydrolysis). In previous studies, *Hd* contained a higher proportion of molecules lower than 1 kDa than did either *H* or *Id* [9], thus suggesting that gastrointestinal digestion after alcalase hydrolysis produced the appearance of very small peptides with lower antioxidant capacity than the parent peptides.

ESR studies with AAPH-derived radicals gave results somewhat different from those of the ORAC assays. Dose–response curve for *Id* sample did not exhibit so marked differences with respect to *I* curve (ESM 1b). Gastrointestinal digestion of *I* produced only about a 26 % diminution in the IC_{50} value, whereas the decrease in the corresponding IC_{50} value effected by alcalase hydrolysis was some 38 %, with *H* having the highest potency ($p < 0.01$; ESM 1b, Table 1). Gastrointestinal digestion of *H*, however, decreased the peptides' scavenging activity, with *Hd* exhibiting an IC_{50} value similar to that of *I* ($p > 0.01$; Table 1). When the scavenging of AAPH-derived radicals is evaluated by ESR, the trapped species depends on the experimental conditions and the spin trap used. Adducts between the common spin traps and ROO· are usually too unstable to be detected, while RO· or R·are detected through the use of diverse traps, with PBN being one of the most frequently employed. Under our experimental conditions, the signal obtained (ESM 2a) can be tentatively assigned to an RO· -PBN adduct according to the splitting constants (a_N=15.5 and a_H=3.7 gauss) [19, 25]. This identification would explain the difference in the results from those obtained with the ORAC assay since the radical species scavenged by each of the two methods would be different: ROO· by the ORAC assay and RO· by ESR. On the basis of these observations, we can hypothesize that the amaranth peptides

Table 1 IC$_{50}$ values for each antioxidant capacity assay for *I, Id, H, Hd,* and reference compounds

Sample	ORAC IC$_{50}$ (mg/ml)	ESR - RO· IC$_{50}$ (mg/ml)	HORAC IC$_{50}$ (mg/ml)	ESR - OH· IC$_{50}$ (mg/ml)	O2·⁻ IC$_{50}$ (mg/ml)	NO· IC$_{50}$ (mg/ml)	ONOO⁻ IC$_{50}$ (mg/ml)
I	0.12 [a] (0.10–0.16)	1.26 [a] (1.12–1.42)	2.29 [a] (2.07–2.54)	4.02 [a] (3.33–4.86)	nc	nd	3.69 [a] (2.94–4.63)
Id	0.024 [b] (0.023–026)	0.93 [b] (0.85–1.02)	0.97 [b] (0.82–1.14)	0.93 [b] (0.87–0.99)	nc	2.85 [a] (2.32–3.49)	0.73 [b] (0.68–0.80)
H	0.024 [b] (0.023–026)	0.78 [c] (0.71–0.85)	1.29 [b,c] (1.09–1.52)	5.11 [a] (4.53–5.76)	nc	3.75 [a] (2.57–5.48)	0.93 [c] (0.83–1.03)
Hd	0.033 [c] (0.031–036)	1.33 [a] (1.21–1.46)	1.31 [c] (1.18–1.45)	2.20 [b] (1.84–2.63)	2.76 (2.39–3.18)	2.85 [c] (2.51–3.24)	1.02 [c] (0.93–1.12)
Ref.	0.007 [a] (0.006–0.0075)	0.019 [a] (0.017–0.022)	0.27 [b] (0.25–0.28)	0.53 [b] (0.51–0.57)	0.10 [c] (0.09–0.11)	3.54 [c] (3.17–3.95)	0.015 [d] (0.013–0.017)

Ninety five percent confidence interval is shown between parentheses. Different superscript letters in the same column indicate significant differences between samples ($p<0.01$)

nr 50 % inhibition was not reached

nc not calculated due to lack of fit of the curves

nd not determined due to experimental problems

Ref. reference compounds

[a] Trolox

[b] Chlorogenic acid

[c] Ascorbic acid

[d] Galic acid

released by gastrointestinal digestion would possess a pronounced activity against peroxyl radicals, but a much less effective scavenging ability with alkoxyl radicals.

Hydroxyl-Radical–Prevention Capacity The HORAC assay—a fluorometric determination to assess hydroxyl-radical inhibition—was optimized with our samples because this method had previously been used to evaluate only phenolic antioxidants [12, 13]. Therefore, dose–response curves were obtained from soluble fractions of the samples (ESM 3a). Although alcalase and gastrointestinal digestion produced an increment in the HORAC activity, this effect was not as pronounced as was seen with the ORAC assay. The IC$_{50}$ value of sample *Id* was about 58 % lower than that of *I*, while the corresponding diminutions for *H* and *Hd* were about 46 and 43 % of the value for *I*, respectively, with no significant differences between them ($p<0.01$) (ESM 3a, Table 1). Although the same fluorescent probe (fluorescein) was used, the concentration ranges for protein activity were quite different from those of the ORAC assay, with the latter enabling the evaluation of much lower amounts of protein. According to a previous report [12], the HORAC assay measured the capacity of antioxidants to chelate metals, thus inhibiting the formation of the OH· radicals; whereas the ORAC method evaluated the capacity of antioxidants to scavenge peroxyl radicals. Thus, a chain-breaking antioxidant like Trolox did not exhibit any activity in this assay (data not shown), and a phenolic acid (chlorogenic acid) had to be used as a reference compound. In addition, those same authors [12] demonstrated that phenolics acted as metal chelators through coordination with Co^{++} and therefore blocked the sites for interaction with H$_2$O$_2$, so as to avert hydroxyl-radical formation. In another study [26], the digestion of buckwheat proteins by pepsin and pancreatin increased the OH· -scavenging activity in an assay similar to those used here, with Trolox as the reference compound. The authors postulated that, in those assays, the antioxidant capacity was expressed as both an inhibition of radical initiation through metal chelation and an elimination of the already formed radicals; they concluded that the inhibition of radical initiation was probably not the critical action determining the radical-elimination efficacy of a hydrolysate. Our results with amaranth proteins, though, suggested that both alcalase hydrolysis and gastrointestinal digestion released peptides with chain-reaction-halting and, to a lesser extent, chelating activities. Nevertheless, gastrointestinal digestion after alcalase hydrolysis failed to produce additional positive radical-combating effects.

As another way to evaluate the activity of amaranth peptides in eliminating OH· radicals, ESR studies were performed. Typical DMPO-OH· -adduct signals are presented in ESM 2b. After gastrointestinal digestion of *I*, the radical-elimination potency of the resulting *Id* peptides became increased by more than 4-fold (ESM 3b, Table 1). This increase

was much greater than that recorded for the same radicals by the HORAC assay. The alcalase hydrolysate H possessed a very low radical-scavenging potency, but this parameter became markedly increased after gastrointestinal digestion (Hd), although the activity of the digest was still significantly lower ($p<0.01$) than that of Id (ESM 3b, Table 1). The OH· -radical–scavenging capacity measured by ESR indicated differences from the HORAC assay in the activity of each sample. This discrepancy is likely related to the mechanism of action evaluated by each assay. As mentioned above, the HORAC assay measures mainly the capacity to chelate metals and thus prevent the formation of OH· radicals [12]; whereas the decrease in the signal intensity of DMPO-OH· in ESR measurements is usually associated with the scavenging of radical species [27]. On the basis of these results, preparations I and H would likely have a lower ability to quench radicals, with the chelating ability being greater than the scavenging activity for both samples. Id, however, demonstrated the greatest ability to inhibit both the formation and the neutralization of OH· radicals. In this regard, a tryptic hydrolysate obtained from jumbo-squid-skin gelatin was able to scavenge OH·, as evidenced by ESR spectroscopy (a 70 % scavenging of the radical at a concentration of 400 μg/mL) [28]. Likewise, a pepsin hydrolysate of silver-carp protein (degree of hydrolysis=20 %) at a concentration of 2 mg/mL produced a substantial reduction of the ESR signal for OH· radicals [29]. These results are comparable to those obtained in the present work; especially with respect to Id, which preparation produced a scavenging ability higher than 70 % at concentrations of about 2 mg/mL (ESM 3b).

Superoxide-Radical–Scavenging Capacity ESM 4a shows the dose–response curves for O_2·⁻ scavenging. The preparations I and Id exhibited a maximum scavenging capacity (with reductions in O_2·⁻ levels of about 40 and 25 %, respectively) at concentrations of around 2 mg/mL, but with the reductions lessening for samples containing higher protein concentrations. In contrast, the preparation H evidenced percent-scavenging values below 15 % at all protein concentrations (ESM 4a). Finally, the results with preparation Hd were fitted to a sigmoidal dose–response model to obtain an IC_{50} value (Table 1). The data suggested that neither alcalase hydrolysis nor gastrointestinal digestion was able to produce peptide mixtures having a high scavenging capacity for O_2·⁻ radicals. When, however, both treatments were applied; the resulting digest Hd possessed the greatest activity, and one especially evident at the higher protein concentrations. We must point out that the following experimental problems encountered with concentrations above 2 mg/ml, prevented an interpretation of the true activities of preparations I, Id, and Hd. A possible explanation could be the formation of turbidity in the reaction mixtures that therefore increased the optical density. Another possible cause could be related to the presence of

molecules in these samples that directly reduced the NBT, where the system used is based on the reduction of NBT exclusively by O_2·⁻ radicals [30]. In order to determine this possibility, control systems containing only samples and NBT (without O_2·⁻) were processed. These assays accordingly demonstrated the formation of turbidity in mixtures of NBT with either I (at concentrations above 1.5 mg/mL) or H (at concentrations above 6 mg/mL). In contrast, the preparations Id and Hd did not demonstrate such turbidity at any concentration; nor did those systems exhibit an absorbance increment through direct reaction of the samples with NBT (data not shown). Therefore, neither turbidity formation nor direct reduction of NBT could explain the lack of scavenging activity of preparation Id. The differing results with the Hd peptides, however, might be owing to the maximum degree of hydrolysis or the different molecular species present in those preparations, which components include a higher proportion of peptides lower than 1 kDa than in the digest Id [9]. An O_2·⁻-scavenging activity has been previously demonstrated for peptides from other sources. Fractions with different molecular masses separated from soybean-protein hydrolysates exhibited scavenging activities between 24.7 and 85.6 %, with an increase in that capacity occurring upon decreases in molecular weight [31]. Gastrointestinal digests from crowpea proteins (12.5 mg/mL), moreover, exhibited an O_2·⁻-scavenging capacity of 16 %, as revealed by a different assay (*i. e.*, pyrogallol-autoxidation measurement) [32]. In the present work, the scavenging activity of the Hd peptides was about 50 % at a protein concentration of 2.76 mg/mL (Table 1), thus achieving a maximal scavenging level of about 60 % at a concentration of about 5 mg/mL (ESM 4a).

Nitric-Oxide–Scavenging Capacity The measurement of NO· scavenging had to be optimized with respect to the relative amounts of SNP, Griess reagent, and sample necessary for observing the activity of the amaranth peptides. For the preparation I, the scavenging activity (at about 20 % radical inhibition) could be measured for only the lower protein concentration assayed (around 0.3 mg/mL) because of the turbidity in the reaction mixture, especially at concentrations above 1.5 mg/mL (data not shown). In order to account for possible sample interferences, the absorbance values of the controls in the absence of SNP—that is, without NO· generation—were measured for each sample and at each concentration. These mixtures containing I became turbid at concentrations above 1.5 mg/mL (similar to reaction mixtures containing SNP). Preparations H, Id, and Hd showed increased absorbances at their highest concentrations, which additional values were subtracted from the absorbances of the reaction mixtures for the calculation of percent scavenging. ESM 4b shows the dose–response curves for the H, Id, and Hd samples. The three hydrolysates showed statistically similar ($p>0.01$) high IC_{50} values (Table 1) that were also comparable

to the reference value with ascorbic acid. Among the very few reports on the scavenging of NO· by protein hydrolysates in the literature, a fraction with species of molecular weights lower than 2 kDa obtained from a gelatin hydrolysate was found to exhibit NO·-scavenging activity in a concentration-dependant fashion: for example, at a dose of 4 mg/mL, the scavenging was 79 %, a value was similar to that of reduced glutathione at the same concentration [33]. In our studies, the scavenging capacity at this concentration was somewhat lower, at approximately 60 % (ESM 4b).

Peroxynitrite-Anion–Scavenging Capacity The peroxynitrite-anion scavenging of the hydrolysates *Id*, *H*, and *Hd* were all three greater than the activity measured with *I*. The *Id* sample exhibited the lowest IC_{50} ($p<0.01$), at about 20 % of the value for *I* (Table 1; ESM 5). The IC_{50} values for *H* and *Hd* were not statistically different, with both being statistically greater than the figure for *Id* ($p<0.01$). As in the other assays, a previous hydrolysis with alcalase did not improve the antioxidant ability after a subsequent gastrointestinal digestion. This difference in scavenging activity between *Id* and *Hd* is probably related to their different molecular compositions [9], thus suggesting that *Id* represented the best balance between antioxidant, prooxidant, and inactive molecules among the four preparations tested.

Conclusions

In the present work, we have explored the capacity of amaranth peptides generated by *in-vitro*–simulated gastrointestinal digestion to scavenge diverse reactive species that are normally present in the human body. The most active hydrolysate was obtained by the direct digestion of the protein isolate since a prior hydrolysis with alcalase failed to improve the preparations' antioxidant capacity in any of the assays performed and in some instances produced in a negative effect. Peptide formation by this form of protein digestion resulted in a strong increment in the scavenging activity against peroxyl radicals (ORAC assay), hydroxyl radicals (ESR-OH· assay) and peroxynitrites. Among the potential mechanisms of action, these amaranth peptides could inhibit the initiation or propagation of radical reactions by being able to act as metal chelators or by donating a hydrogen atom, although the increment in that former activity by digestion was not so pronounced. That these peptides could be involved in other antioxidant mechanisms in addition to their direct action on the above radical species—such as an induction of the genetic expression of enzymes that can protect the cell from oxidative damage—is highly relevant and merits further consideration. These concerns will be the object of continuing studies in our laboratory.

In conclusion, these results have allowed us to distinguish the most significant mode of direct action on the part of the peptides encrypted in the storage proteins of amaranth grain that are released by simulated gastrointestinal digestion. We have also demonstrated a promising potential capacity of these peptides to prevent oxidative stress in the human body. The complete peptide mixture generated by this means that it—as has been analyzed here—could potentially exert its antioxidant activity in the intestinal tract and/or on the cells of the intestinal villi, but the real ability of these peptides to act inside the human body will depend on their capacity to be absorbed through the intestinal mucosa and thus access that site of action. Studies along those lines are currently being performed in our laboratory in order to completely evaluate amaranth proteins as functional ingredients in foods.

Compliance with Ethics Requirements

Conflict of Interest M. C. Orsini Delgado declares that she has no conflict of interest.

M. Galleano declares that she has no conflict of interest.

M. C. Añón declares that she has no conflict of interest.

V. Tironi declares that she has no conflict of interest.

This article does not contain any studies with human or animal subjects.

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