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# Identification and characterization of antioxidant peptides obtained by gastrointestinal digestion of amaranth proteins

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ABSTRACT

The objective of the present work was to separate and identify antioxidant peptides from a simulated gastrointestinal digest (*Id*) from *Amaranthus mantegazzianus* proteins (*I*), which has previously been demonstrated to have this activity. *I* and *Id* were separated by preparative RP-HPLC. Fractions were evaluated by the ORAC method and the more active ones were analyzed by LC–MS/MS. Each fraction presented diverse peptides from different proteins, most of them from the 11S globulin. After grouping the peptides from 11S globulin according to their overlapping sequences, and based on previous information about structure–activity relationships, ten sequences were synthesized, in order to evaluate their antioxidant activity. Four peptides presented interesting activity: AWEEREQGSR > YLAGKPQQEH ~ IYIEQGNGITGM ~ TEVWDSNEQ. They exhibited some of the structural characteristics already known to demonstrate this activity, all of them containing at least one bulky aromatic residue. All belonged to little structured, internal or exposed regions of the acid subunit of the 11S globulin.



## 1. Introduction

Diverse peptides, which can be *in vitro* or *in vivo* released from animal or plant proteins, have shown potential beneficial effects on health, one of them being the antioxidant capacity (Hartmann & Meisel, 2007; Udenigwe & Aluko, 2012). Mechanisms of action of antioxidant peptides are not completely understood, but some studies have demonstrated that they can be free radicals scavengers, metal transition chelators or lipid peroxidation inhibitors. In addition, some peptides can avoid oxidative damage by induction of the genes that codified for the antioxidant enzymes (Sarmadi & Ismail, 2010). Antioxidant activity of the peptides is related to the protease action and the extension of the hydrolysis process, as well as to the structural characteristics of peptides such as molecular size, hydrophobicity and amino acidic composition (Pihlanto, 2006; Udenigwe & Aluko, 2012). Histidine, cysteine, proline, methionine, lysine and aromatic amino acids have demonstrated antioxidant activity by different mechanisms. As an example, in peptides (3–16 amino acids) from soy proteins, the histidine – and particularly the imidazole group position – were identified as important for the hydrogen-donating, peroxyl radical scavenging and/or the metal chelation activities (Chen, Muramoto, Yamauchi, & Nokihara, 1996). Antioxidant activity of these peptides was enhanced by the presence of hydrophobic amino acids (proline and leucine) in the N-terminus. Chen, Muramoto, Yamauchi, and Nokihara (1998) have postulated that hydrophobic amino acids can increase the accessibility of the antioxidant peptides to hydrophobic cellular targets such as the polyunsaturated chain of fatty acids of biological membranes. Aromatic amino acids tyrosine, phenylalanine and tryptophane can donate protons to free radicals and contribute to the chelating of pro-oxidant metal ions. The SH group of cysteine can also scavenge free radicals. Sarmadi and Ismail (2010) and Udenigwe and Aluko (2012) have performed an extensive revision of antioxidant peptides and their mechanisms of action.

Antioxidant properties of amaranth (*Amaranthus mantegazzianus*) proteins and peptides have been studied in our lab. In this way, we have demonstrated the presence in the amaranth seeds of naturally-occurring peptides and polypeptides with free radical scavenging and inhibition of the linoleic acid oxidation activities; these are active molecules distributed into the different protein

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fractions (albumins, globulins and glutelins). Alcalase hydrolysis was able to improve the scavenging activity of both the isolate and the protein fractions by causing the release of small peptides and/or free amino acids with such activity (Tironi & Añón, 2010). In addition, a simulated gastrointestinal digestion also improved the antioxidant activity of amaranth isolates and hydrolysates, suggesting a potential *in vivo* release of active peptides (Orsini Delgado, Tironi, & Añón, 2011). Gastrointestinal digests have demonstrated the ability to act through different reactive species present in the human body, especially against peroxyl and hydroxyl radicals and peroxynitrites (Orsini Delgado, Galleano, Añón, & Tironi, 2015).

Taking into account that: (1) amaranth proteins contain encrypted peptides that can be release by enzymatic hydrolysis, with potential antioxidant activity which has been demonstrated by *in vitro* studies; (2) antioxidant activity is related to composition and structural characteristics of the peptides; (3) to date amaranth peptide sequences with antioxidant activity have not been reported; the aim of the present work was to study the identity of amaranth antioxidant peptides as well as their structure–activity relationships, in order to evaluate possible ways of preparation and incorporation of them into a food matrix.

## 2. Materials and methods

### 2.1. Chemicals

Alcalase 2.4L (protease of Bacillus licheniformis, Novozyme Corp), and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pepsin 1:15000 5X NF standards and porcine pancreatin 4X-100USP units/mg were from MP Biomedicals LLC (Solon, OH, USA). Fluorescein sodium was from Fluka (Steinheim, Germany) and AAPH (2,2′-Azo-bis - (2-methylpropionamidine) dihydrochloride) was from Aldrich (Wisconsin, USA). Acetonitrile was of HPLC grade while all of the other reagents were of analytical grade.

### 2.2. Samples

#### 2.2.1. Protein isolate (I)

*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, unpeeled, seeds in an Udy mill, 1 mm mesh, screened by 0.092 mm mesh, and defatted by extraction with hexane for 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. Simulated gastrointestinal digestion

The simulated digestion has been previously optimized for amaranth proteins (Orsini Delgado et al., 2011). Briefly, the sample (*I*) was initially treated with a pepsin solution (0.1 equi/L HCl, 0.03 mol/l NaCl, pH = 2) using a pepsin/protein ratio of 1/10 w/w, at 37 °C with agitation, for 60 min. Then, the pH was adjusted to 6 and a pancreatin solution (0.1 mol/l $NaHCO_3$, pH = 6) was added (pancreatin/protein ratio = 1/10 w/w), followed by incubation at 37 °C with agitation for 60 min. Control reactions, without enzymes added, were performed. Pancreatin activity was stopped by heating at 85 °C for 10 min and suspensions were freeze-dried. Digest was named *Id*. Hydrolysis degree (HD) measured by the 2,4,6-trinitrobencenesulfonic acid (TNBS) method (Adler-Nissen, 1979; Orsini Delgado et al., 2011) was 36.9 ± 0.5%.

#### 2.2.3. Soluble fractions preparation

10 mg/ml suspensions from freeze-dried samples (*I* and *Id*) in 32.5 mmol/l $K_2HPO_4$/2.6 mM $KH_2PO_4$ (pH = 7.8) were prepared by agitation at 300 rpm for 1 h at 37 °C, (Termomixer Eppendorf). Suspensions were centrifuged at 10,000*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.

### 2.3. Preparative RP-HPLC

A SunFire prep C8, 5 μm ST 10/250 (Waters) column was used to isolate the components present in the soluble fractions of samples *I* and *Id* on a Waters System HPLC (Waters Corp., Milford, MA) equipped with a diode array detector. Solvent A was a mixture of water and acetonitrile (98:2) with trifluoroacetic acid TFA (650 μl/l), and solvent B was a mixture of water and acetonitrile (35:65) with TFA (650 μl/l). Filtered (0.45 μm) samples were eluted with a linear gradient of solvent B in A (0 to 100% in 55 min, flux rate: 5.2 ml/min). Separation was carried out at 40 °C. Detection was performed at 210 and 280 nm. Fractions (0.6 min) were collected, pooled and freeze-dried. Runs were performed by duplicate.

### 2.4. Mass spectrometry

#### 2.4.1. General

Mass-spectrometry analyses were conducted by the platform "Biopolymers-Structural Biology" located at the INRA Center of Angers-Nantes, France (http://www.angers-nantes.inra.fr/plateformes_et_plateaux_techniques/plateforme_bibs).

#### 2.4.2. LC–MS/MS analysis

Freeze-dried samples were resuspended in 30 μl of solvent A (0.1% HCOOH in water), diluted and analyzed by nanoflow liquid chromatography-tandem mass spectrometry (nanoLC–MS/MS). These experiments were performed on a LTQ-Orbitrap VELOS mass spectrometer (Thermo-Fisher) coupled to a nanoscale liquid-chromatography (LC) system (U3000 RSLC system, Thermo-Fisher). LC separation was performed on a 15-cm long reverse-phase capillary column (Acclaim Pepmap C18 2-μm 100 A, 75-μm i.d.) using a 28-min linear gradient from 4% to 50% (v/v) solvent B (90% acetonitrile 0.08% formic acid) in solvent A (0.1% HCOOH in water). The nanoflow rate was set at 300 nL/min. A typical survey method was used for the fragmentation of the peptides (MS/MS), in which full MS scans were acquired at 30,000 resolution (FWMH) using the Orbitrap analyzer (on a m/z range of 300–2000) while the collision-induced dissociation (CID) spectra (MS/MS) for the five most intense ions were recorded in the linear LTQ trap.

#### 2.4.3. Protein identification

All fractions were analyzed separately on the mass spectrometer but analytical results were combined to improve proteins identification in the databank. Protein identification was achieved by comparing mass data against the UniProt databank restricted to the *Amaranthus* taxonomy, using MASCOT Server 2.2 (Matrix Science). The parameters used for database searches included: variable oxidation of methionins, and tolerance of the ions at 5 ppm for parents and 0.5 Da for fragments. Proteins were considered as valid when they were matched with one unique peptide