Amaranth peptides with antithrombotic activity released by simulated gastrointestinal digestion

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ABSTRACT

Amaranth protein isolate was obtained and subjected to simulated gastrointestinal digestion to evaluate its potential antithrombotic activity. The protein isolate did not present fibrin clotting inhibition at the concentrations studied, whereas the hydrolysate (DH% = $51.1 \pm 3.8\%$) exhibited inhibition of fibrin coagulation, showing a dose–response behaviour ($IC_{50} = 0.23 \pm 0.02$ mg/mL), confirming that the enzymatic treatment was able to release bioactive peptides from amaranth proteins. A fraction with high antithrombotic activity was obtained from this hydrolysate, and resulted to be three times more potent than its original sample ($IC_{50} = 0.07 \pm 0.01$ mg/mL). The absorption of this active fraction was studied with an *in vitro* peptides transport assay through intestinal epithelium and it was observed that some peptides are able to cross the Caco2-TC7 cell monolayer. Potentially bioactive peptides were found after sequencing them, and informatics tools allowed us to select and locate in their native molecules those peptides prone to inhibit thrombin activity.

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1. Introduction

Many diseases prevalent in the world nowadays present an important relation with the diet. A healthy diet combined with the intake of functional foods may help minimize or even prevent certain diseases (Jew, AbuMweis, & Jones, 2009). The functional components can be diverse molecules or microorganisms; among them are bioactive peptides which generally present short sequences and are encrypted in food proteins (Quian, Jung, & Kim, 2008; Udenigwe & Aluko, 2012). Over the last decade there have been described different biological activities performed by the bioactive peptides: antimicrobial, antiproliferative, antioxidant, antihypertensive, hypocholesterolaemic, and antithrombotic activity among others (de Castro & Sato, 2015; Hartmann & Meisel, 2007). While some of them act on the gastrointestinal system, others require the absorption and transport of peptides into specific sites where they will exercise their action

(Satake et al., 2002). Bioactive peptides could be released from food proteins due to the action of microorganisms' metabolism during fermentation, proteolytic enzymes during food processing or the action of gastrointestinal enzymes once proteins are ingested. An approach to the latter route would be simulated gastrointestinal digestion, a process that promotes the formation of active peptides (Hernández-Ledesma, Amigo, Ramos, & Recio, 2004) and sometimes increases bioactivity of peptides (Orsini Delgado, Tironi, & Añón, 2011). This mechanism of peptides production has as an additional advantage on the fact that it checks the stability of the potentially active peptides through the digestive system (Miguel, Aleixandre, Ramos, & López-Fandiño, 2006). Peptides contained in amaranth seed proteins have shown various biological activities (Barrio & Añón, 2009; Caselato-Sousa & Amaya-Farfán, 2012; Fritz, Vecchi, Rinaldi, & Añón, 2011; Huerta-Ocampo & Barba de la Rosa, 2011; Mendonça, Saldiva, Cruz, & Arêas, 2009; Orsini Delgado et al., 2011; Quiroga, Barrio, & Añón, 2015; Silva-Sánchez et al., 2008; Tiengo, Faria, & Netto, 2009). The seeds of this Mesoamerican pseudocereal present high percentage of proteins with an excellent amino acid balance (Bale & Kauffman, 1992). Moreover, amaranth easily adapts to conditions unfavourable for other crops (Paredes-López, 1994).

Antithrombotic peptides inhibit the fibrinogen binding to a specific receptor region on the platelet surface and also inhibit aggregation of platelets. Several antithrombotic peptides derived from food proteins have been described over the last years. Many of them are contained in the milk protein κ-casein, as they exhibit sequence homologies with fibrinogen chains and bind to a specific receptor region on the platelet surface (Hayes, Stanton, Fitzgerald, & Ross, 2007). The presence of antithrombotic peptides in other food sources, such as velvet bean, rapeseed and egg white (Segura-Campos, Tovar-Benítez, Chel-Guerrero, & Betancur-Ancona, 2014; Yang, Wang, & Xu, 2007; Zhang, Wang, & Xu, 2008) has also been described. Recently, *in vitro* antithrombotic activity was observed in amaranth hydrolysates and protein fractions (Sabbione, Scilingo, & Añón, 2015), but no bioactivity was found in the protein isolate. These amaranth hydrolysates were able to inhibit the thrombin enzyme (EC 3.4.21.5), which is a serine protease that participates at the end of the coagulation cascade and proteolyses fibrinogen to generate fibrin monomers that subsequently polymerize.

The main objectives of this work were to determine if proteins from amaranth manifest antithrombotic activity after subjected to simulated gastrointestinal digestion, and to find those sequences that potentially inhibit thrombin, with the final purpose of using amaranth isolates as functional ingredients.

2. Materials and methods

2.1. Plant material and samples preparation

2.1.1. Amaranth seeds
Amaranthus hypochondriacus (cv Mercado) seeds were harvested in 2011 at Río Cuarto, Córdoba, Argentina.

2.1.2. Amaranth flour preparation
Seeds were ground with a cyclone mill (1 mm mesh) and the resulting flour was defatted with n-hexane (10 g flour/100 mL

n-hexane) during 24 h, the five first hours with constant stirring and then overnight contact.

2.1.3. Preparation of protein isolates
Amaranth protein isolate was obtained as described by Martínez and Añón (1996).

2.1.4. Preparation of hydrolysates
The method used to simulate gastrointestinal digestion of amaranth isolates was an adaptation of published methods (Hernández-Ledesma et al., 2004; Roesler & Rao, 2001). The protein isolate was suspended in saline solution (pH 2, HCl 0.1 mol/L, 0.03 mol/L NaCl) and treated with a pepsin solution (0.1 g pepsin/g protein, 1:15,000 5X NF units, MP Biomedicals, Santa Ana, CA, USA) at 37 °C with agitation during 1 h. Then, pH was adjusted to 6 and a pancreatin solution was added (0.1 g pancreatin/g protein, 4X-100 USP units/mg, MP Biomedicals, 0.1 mol/L NaHCO₃), incubating at 37 °C with agitation during 1 h. Finally, pancreatin activity was stopped by heating at 85 °C for 10 min and suspensions were freeze-dried.

2.2. Samples characterization

2.2.1. Centesimal composition
Protein content was determined by Kjeldahl, using the conversion factor 5.85 g protein/g nitrogen (Paredes-López, 1994). Carbohydrates content was determined by the anthrone method (Yemm & Willis, 1954) after complete acid hydrolysis. Ash content was obtained by heating in a muffle at 550 °C, and water content by drying in a stove at 105 °C until constant weight is attained (AOAC methods 923.03, and 925.09; 1990). Fibre content was calculated by difference.

2.2.2. Tricine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (tricine SDS-PAGE)
Tricine-SDS-PAGE was performed as described by Schägger (2006). Runs were carried out in stacking, spacing and separating gels (160, 100 and 40 g/L acrylamide respectively) with 2-mercaptoethanol (2-ME, 50 mL/L sample buffer). Two protein molecular mass standards were used: GE Healthcare (Uppsala, Sweden) low molecular standards and Bio-Rad (Hercules, CA, USA) very low molecular standards. Gels were fixed in a methanol–acetic acid solution and stained with Coomassie Brilliant Blue R-250 (Anedra, San Fernando, Argentina) or when necessary, silver staining was applied to increase analytical sensitivity.

2.2.3. Degree of hydrolysis (DH%)
Free amino groups were quantified by the o-phthaldialdehyde method (OPA, Sigma, St. Louis, MO, USA) according to Nielsen, Petersen, and Dambmann (2001). To calculate the DH%, the following expression was used:

$$DH\% = 100 \times (NH_{2\,t} - NH_{2\,t0})/(NH_{2\,t\infty} - NH_{2\,t0}) \qquad (1)$$

$NH_{2\,t}$, $NH_{2\,t0}$, and $NH_{2\,t\infty}$ were the free amino groups at time t, initial time (0) and infinite time of hydrolysis, respectively. $NH_{2\,t0}$ was determined experimentally from an unhydrolysed

isolate, and $NH_{2\,t\infty}$ was obtained using the following expression:

$$NH_{2\,t\infty} = (1/MW_{aa}) \times (1 + f_{Lys}) \times P \times 1000 \qquad (2)$$

where MW_{aa} is the average molecular weight of the amino acids of amaranth proteins, 130 g/mol, f_{Lys} is the lysine proportion in amaranth proteins, 1/16 (Paredes-López, 1994) and P is the protein concentration.

2.3. Antithrombotic activity

2.3.1. Samples preparation
Suspensions of the samples in the assay buffer (50 mmol/L Tris–HCl, pH 7.2, 0.12 mmol/L NaCl) were prepared, and incubated for 1 h at 25 °C with agitation. Samples were then centrifuged at 10,000 g for 30 min at 15 °C. The protein content in the supernatant was determined by the Lowry colorimetric method (Lowry, Rosebrough, Farr, & Randall, 1951).

2.3.2. Determination
Antithrombotic activity was determined with the microplate method informed by Yang et al. (2007). A range of concentrations of each solubilized sample was studied. In a microplate 140 µL of 0.1 g/100 mL fibrinogen (Sigma) were added, 40 µL of every sample or buffer as a negative control of the coagulation inhibition or 0.3 mg/mL heparin (Abbott, Alameda, CA, USA) as a positive control of the coagulation inhibition. The coagulation process was initiated after adding 10 µL of thrombin (12 UI/mL, Sigma). Absorbance at 405 nm was measured in a microplate reader (BioTek Synergy HT, Winooski, VT, USA) before adding the enzyme and after 10 min incubation at 37 °C. The percentage of inhibition (% Inhibition) was calculated with the following equation:

$$\% \text{ Inhibition} = 100 \times [(C - CB) - (S - SB)]/(C - CB) \qquad (3)$$

where,

CB (control blank):	the initial absorbance of the negative control of inhibition.
C (control):	the absorbance of the negative control at 10 min of incubation with thrombin.
SB (sample blank):	the initial absorbance of the sample.
S (sample):	the absorbance of the sample at 10 min of incubation with thrombin.

2.4. Obtaining bioactive peptides

Fractionation of the hydrolysate was performed using size-exclusion chromatography (SEC) in a Pharmacia LKB, FPLC System, using a molecular exclusion column (GE Healthcare total volume 25 mL), Superdex-75 10/300-GL column (range: 3–70 kDa). The column was calibrated with blue dextran (void volume, Vo = 7.3 mL), albumin (67 kDa), ovalbumin (44 kDa), chymotrypsin (25 kDa), ribonuclease (19 kDa) and aprotinin (6.5 kDa) from GE Healthcare, obtaining the following calibration equation:

$$\log MW = -0.1104 \, Ve + 2.701 \qquad (4)$$

where MW and Ve are molecular weight and elution volume of the resolved species, respectively.

2.5. Absorption in vitro assay

2.5.1. Caco 2 cell culture
Caco 2-TC7 cells were obtained from American Type Culture Collection (Rockville, MD, USA) and passage numbers between 22 and 30 were used for the formation of the monolayer. Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, inactivated foetal bovine serum (FBS) and antibiotics (1000 UI penicillin; 1000 µg/mL streptomycin), pH 7.4 was purchased from Microvet SRL (CABA, Argentina) and sterilized with 0.22 µm pore size filter (Merck Millipore, CABA, Argentina).

Caco 2-TC7 cells were cultured using techniques described by Yamashita et al. (2000) and Yamashita et al. (2002) at 37 °C, 5% CO_2 atmosphere, and 90% relative humidity. Polycarbonate inserts were seeded (Corning, NY, USA, 0.65 mm diameter; 0.4 µm pores; 24 wells/plate) at a density of 1×10^5 cells/cm², to obtain a uniform monolayer. Medium was replaced every 48 h and the transepithelial electrical resistance (TEER) was measured routinely with a volt ohmmeter equipment (Millicell ERS system, Millipore, Billerica, MA, USA) to analyse the confluence and integrity of the monolayer formed in the inserts. The transepithelial transport activity was determined in apical-basolateral direction using those monolayers that presented TEER values between 450 and 550 ohms.cm² in the inserts, as described by Yamashita et al. (2000, 2002). Fig. 1 shows the inserts and geometry described.

2.5.2. Peptides transport study
Caco 2-TC7 were grown in transwell inserts and placed in 24-well acrylic plate; phosphate buffered saline (PBS) was added in apical and basolateral chambers (0.5 mL) and plates were incubated at 37 °C, 5% CO_2 and 90% relative humidity for 20 min. After incubation time PBS was collected and 0.2 mL of samples were placed in the apical chamber of the inserts, whereas 0.65 mL of PBS was placed in the basolateral chamber (3 h, 37 °C, 5% CO_2 and 90% relative humidity). Finally, volumes of apical and basolateral chambers were collected for further analysis using RP-HPLC.

Samples used for the transepithelial transport assay were diluted with serum-free DMEM to prepare test solutions.

2.5.3. Analytical methods
The content of apical and basolateral chambers (100 µL) were analysed with a reversed-phase HPLC system (Waters, Milford, MA, USA), analytical column Sephasil Peptide C8, 12 µm ST 4.6/250 (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The volume eluted was collected in fractions of 1.1 mL/tube during the first 60 min of the chromatographic runs.

2.5.4. Cytotoxicity
Methyl tetrazolium assay (MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) was carried out as described by Di Virgilio, Reigosa, Arnal, and Fernández Lorenzo de Mele (2010).
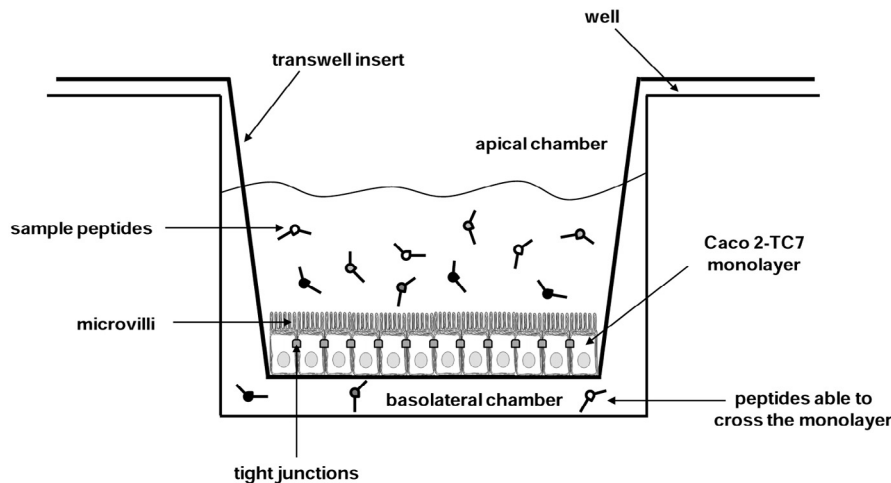
Fig. 1 – Experimental scheme employed to study *in vitro* absorption of peptides.

Neutral red assay was performed according to Borenfreund and Puerner (1984).

2.6. Mass spectrometry

Mass-spectrometry analyses were conducted by the platform "Biopolymers – Structural Biology" located at the INRA Center of Angers-Nantes, France. MALDI-TOF analysis (Micromass/ Waters) was performed to check the sample complexity and estimate the mass of the peptides: 1 µL of the sample was mixed with 1 µL of the matrix preparation (2.5 g/L α-cyano-4-hydroxycinnamic acid, 2.5 g/L 2,5-dihydroxybenzoic acid, 70% v/v acetonitrile, and 0.1% w/v trifluoroacetic acid) and deposited onto the MALDI sample probe. Many masses below 4000 Da were found; hence an LC–MSMS analysis was performed to get the peptide sequences.

2.6.1. LC–MS/MS analysis
Freeze-dried samples were suspended in 0.1% formic acid in water (solvent A), diluted and analysed by nanoflow liquid chromatography–tandem mass spectrometry (LC–MS/MS). These experiments were performed on an LTQ-Orbitrap VELOS mass spectrometer (Thermo Fisher, Waltham, MA, USA) coupled to a nanoscale liquid-chromatography (LC) system (U3000 RSLC system, Thermo Fisher). Chromatographic separation was conducted on a reverse-phase capillary column (Acclaim PepMap C18, 2 µm particle size, 100 Å pore size, 75 µm i.d., 15 cm length, LC Packings) using a 28 min linear gradient from 4 to 50% v/v 90% acetonitrile, 0.08% formic acid (solvent B) in solvent A, at a flow rate of 300 nL/min. Fragmentation of the peptides (MS/ MS) was acquired with full MS scans at 30,000 resolution (FWMH) using the Orbitrap analyser (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.

2.6.2. Protein identification
Protein identification was achieved by comparing the collected LC–MS/MS data against the Uniprot databank restricted to the *Amaranthus* taxonomy, using MASCOT Server 2.2 (Matrix Science, Boston, MA, USA). The parameters used for database searches included: variable oxidation of methionines, and a mass tolerance of 5 ppm for parent ions and 0.5 Da for fragments. Proteins were considered as valid when they were matched with one unique peptide with an individual score above the significance threshold score computed by MASCOT ($p = 0.05$).

2.7. Bioinformatic tools

BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) was used as the reference database to consult already identified antithrombotic peptides (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008). MEROPS database (http://merops .sanger.ac.uk) provided thrombin specificity information (Rawlings, Waller, Barrett, & Bateman, 2014). ExPASy peptide cutter tool (http://web.expasy.org/peptide_cutter) was used to simulate the proteolysis employed (Gasteiger et al., 2005). Images and analysis of the location of identified peptides in their molecules was performed with The PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC.

2.8. Statistical analysis

Isolates and protein hydrolysates were prepared several times (two at least). Results were evaluated statistically by using variance analysis (ANOVA) with the *post-hoc* LSD Fisher test ($p = 0.05$).

3. Results and discussion

3.1. Samples characterization

The per cent composition of the amaranth protein isolate and its hydrolysate was determined (Table 1). The protein percentage of the isolate was similar to values previously informed (Condés, Scilingo, & Añón, 2009; Orsini Delgado et al., 2011). Fibre content, estimated by difference, doubled the value informed by Condés et al. (2009), probably due to the fact that

Table 1 – Centesimal composition and degree of hydrolysis (DH%) of the isolate (I) and hydrolysate obtained after simulation of the gastrointestinal digestion (H).

	Centesimal composition (g/100 g sample)					DH%
	Proteins	Carbohydrates	Ashes	Water	Fibres[*]	
I	71.3 ± 1.5^b	9.0 ± 0.8^b	3.6 ± 0.1^a	6.4 ± 0.6^a	9.7	–
H	55.5 ± 0.9^a	5.3 ± 1.0^a	17.4 ± 0.1^b	12.5 ± 1.4^b	9.3	51.1 ± 3.8

Different superscript letters in the same column corresponded at different values ($p = 0.05$, LSD).
[*] Fibre content was obtained by difference.

samples in this work were obtained from non-sifted *A. hypochondriacus* flour. Significant differences are shown in Table 1 between the isolate and its hydrolysate when analysing centesimal composition. Minerals content of the hydrolysate is approximately five times greater than the isolate owing to successive additions of buffers for the simulated gastrointestinal digestion. The hydrolysate retained more water during the lyophilization or it was partially hydrated in the handling due to the higher hydrophilicity of the sample. Degree of hydrolysis (DH%) of the hydrolysate was 51.1 ± 3.8%, significantly higher in comparison with the DH% reached when hydrolysing amaranth proteins with commercial proteases (Sabbione et al., 2015), indicating that once ingested, the isolate would undergo extensive hydrolysis. Orsini Delgado et al. (2011) obtained a lower DH%, approximately 40%, using the same digestion protocol.

3.2. Antithrombotic activity

In vitro antithrombotic activity of the samples was evaluated with the microplate method in which ability to prevent formation of the fibrin clot is analysed by looking at a diminution or total absence of turbidity. The assay emulates the last stage of blood coagulation, where thrombin hydrolyses fibrinogen generating fibrin monomers that subsequently polymerize. The isolate did not inhibit coagulation at the concentrations studied (0.17 to 12.0 mg protein/mL) coinciding with what informed Sabbione et al. (2015). The hydrolysate exhibited a coagulation inhibition curve vs protein concentration, showing a dose–response behaviour (Fig. 2a). Inhibitory activity of the hydrolysate was observed at 0.2 mg/mL, and higher concentrations increased the inhibition reaching a *plateau* at approximately 0.8 mg/mL. These data were processed with the GraphPad Prism program to calculate the IC_{50} of the sample (concentration that inhibits the 50% of the thrombus formation), which resulted to 0.23 ± 0.02 mg/mL. This IC_{50} value verifies the effectiveness of the digestion to produce peptides with potential antithrombotic activity. Moreover, IC_{50} values of the hydrolysate resulted lower than those informed for many amaranth protein fractions and its hydrolysates, and slightly higher than glutelin fraction (Sabbione et al., 2015). Other authors informed potential antithrombotic activity of different hydrolysed food proteins. While Shimizu et al. (2009) found that pork peptides could be beneficial in preventing thrombosis, Yang et al. (2007) informed IC_{50} values of 50 mg/mL for egg white peptides, and Zhang et al. (2008) IC_{50} of 30 mg/mL for rapeseed peptides. The high activity of the hydrolysate could be

associated with the degree of hydrolysis reached after performing simulated gastrointestinal digestion, thus, with the presence of small peptides. However, the fact that specific inhibitory peptides are generated cannot be discarded. The potential inhibition of the hydrolysate is a promising result, as the isolate could be the product used as a biologically active food ingredient, and gastrointestinal digestion could be the releasing mechanism of antithrombotic peptides. Moreover, the released peptides are able to resist gastrointestinal digestion. This is a desirable characteristic of bioactive peptides (Korhonen & Pihlanto-Leppälä, 2001; Picariello et al., 2010).

To separate active compounds of the hydrolysate, a size-exclusion chromatography was used with a column that separates mass species between 3 and 70 kDa. The hydrolysate was solubilized in buffer Tris–HCl 0.05 mol/L, NaCl 0.12 mmol/L, pH 7.2 and eluted with the same solvent. A fraction at elution volume of 14–16 mL was the only one that presented clot formation inhibition using the microplate method (Fig. 2b). The antithrombotic activity of this active fraction (AF) was studied at different concentrations. Fig. 2c shows the dose–response inhibition curve obtained as the protein concentration of the sample increases. AF inhibition of the clot formation started at 0.03 mg/mL protein, and was able to generate a total inhibition at 0.17 mg/mL protein. The IC_{50} value was 0.07 ± 0.01 mg/mL, indicating that AF is three times more potent than its original sample, the hydrolysate.

Fig. 2d exhibits an electrophoretic Tricine-SDS-PAGE gel. The profile of the *A. hypochondriacus* isolate was similar to that previously described by Sabbione et al. (2015). The hydrolysate also presents a profile where bands between 26 and 20 kDa stand out and numerous bands under 14.4 kDa appear continuous (Fig. 2d, H). The profile of AF preserves the sharpest band observed in the hydrolysate (22 ± 1 kDa) with other low molecular weight bands of very low intensity.

3.3. Absorption in vitro assay: peptides transport study

To analyse whether peptides content in AF are able to overcome intestinal barriers, an *in vitro* assay was performed. Peptides were faced with a Caco 2-TC7 epithelial cell monolayer using devices called transwell inserts (Fig. 1). Cytotoxicity tests, MTT and neutral red had to be done to study if AF had an effect on the viability of cells. It was observed that cells preserved 100% viability in both assays, showing no cytotoxicity effect of the sample over Caco 2-TC7 cells at the concentrations studied (highest concentration: 0.81 ± 0.03 mg/mL protein), maintaining total integrity of the monolayers.
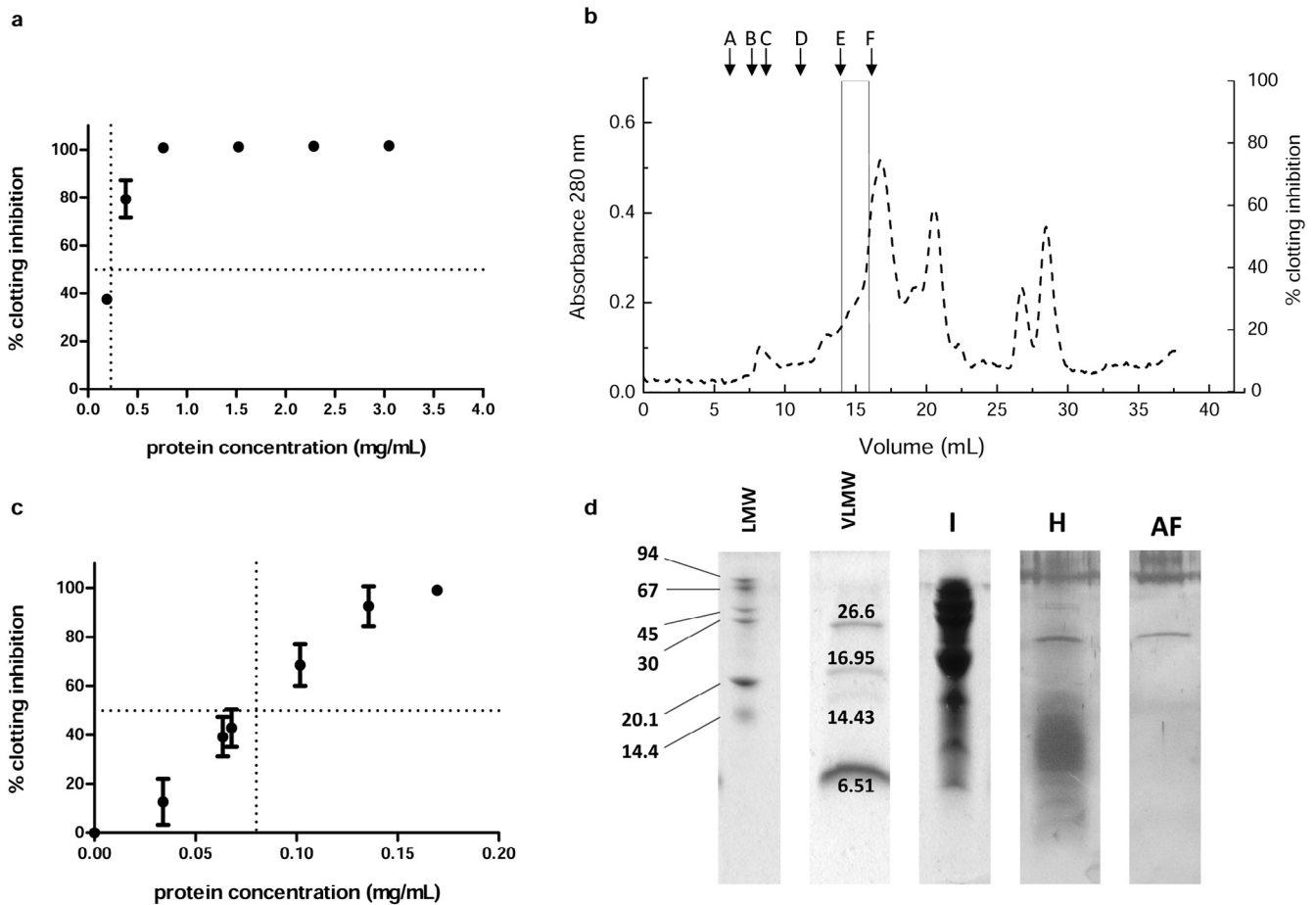
Fig. 2 – (a and c) Percentage of inhibition of thrombus formation vs protein concentration of amaranth hydrolysate (H) and active fraction (AF) respectively. Error bars refer to standard deviation. (b) SEC chromatogram of amaranth hydrolysate (H, —-) fractioned using Superdex 75 column (range 3–70 kDa, standard calibration: albumin (B), ovalbumin (C), chymotrypsin (D), ribonuclease (E) and aprotinin (F)). Exclusion volume: blue dextran (A). Per cent clotting inhibition of the fractions collected is exhibited (—). (d) Tricine-SDS-PAGE in reducing conditions. Amaranth isolate (I), hydrolysate (H), and active fraction (AF). LWM and VLMW: low and very low standards molecular weight.

After the absorption *in vitro* assay of AF, volumes of apical and basolateral chambers were collected from the inserts. Antithrombotic activity of these samples was studied using the microplate method. While AF inhibited completely the clot formation (0.81 ± 0.03 mg/mL protein), the volume of the apical chamber inhibited $91.6 \pm 2.0\%$ (0.75 ± 0.08 mg/mL protein). The decreased activity of the apical volume after the absorption assay could be associated with the passage of bioactive peptides through the monolayer, in apical-basolateral direction. The antithrombotic activity determined for the basolateral volume was inferior to that obtained for the apical volume, $5.2 \pm 0.5\%$ (peptide concentration not detected with Lowry method). This result would suggest that some species from AF were able to cross Caco 2-TC7 monolayer and to inhibit coagulation, or that cells released inhibitory compounds to the basolateral chamber. A control assay was performed where PBS was added to the apical chamber instead of the sample. Potential inhibition of coagulation was determined in apical and basolateral volumes, and no sign of activity was detected, discarding our second hypothesis.

It must be taken into consideration that in normal situations, intestinal epithelium has high proteolytic activity. Proteases in the enterocytes complete the proteolytic work initiated in the lumen (Langguth et al., 1997); hence, it is possible that the species found in the apical chamber would get modified inside the cells, generating changes in the initial antithrombotic activity. Even though the experimental strategy was to find the same species contained in the AF, there may be other active species in apical and basolateral chambers.

Reversed-phase chromatography HPLC was used to analyse species contained in apical and basolateral chambers after incubation of AF with the monolayer in the inserts. Samples were filtered and injected in the HPLC equipment. Complementary chromatographies of PBS buffer and AF were performed and a comparative analysis of the chromatograms was made. Three peaks were identified when subtracting peaks of the PBS chromatogram from the peaks of the chromatogram obtained when analysing basolateral sample. These three peaks coincided with those in chromatograms of apical sample and

AF (not shown results). The tubes that were collected that contained those peaks were lyophilized and analysed.

3.4. Sequencing and bioactive peptides identification

Sequences of peptides from AF and basolateral chamber were obtained using MALDI-TOF and liquid nanochromatography with mass spectrometry associated (LC–MS/MS). Results were analysed with MASCOT 2.2 program against Uniprot restricted to *Amaranthus* taxonomy.

The MALDI mass spectrum of AF was performed. As many masses presented molecular weights below 4000 Da, liquid nanochromatography coupled to tandem mass detectors (MS/MS) was implemented to obtain sequence data. From all the peaks detected, three were identified on the database due to the fact that only few amaranth proteins are sequenced. *De novo* sequencing was performed to overcome this difficulty, but unreliable results were achieved. The peaks identified in MALDI contained many peptides with sequences belonging to proteins indicated in Table 2. In some cases, various sequences that correspond to the same protein were found, and were pooled in families or groups of sequences that belong to the same region.

Taking into consideration that AF comes from amaranth protein isolate, only sequences found in 11S globulin and agglutinin were analysed, both present in the isolate. Most peptides detected in AF come from 11S globulin, sequenced by Molina, Circosta, Añón, and Petruccelli (2008), and agglutinin or lectin, sequenced by Rinderle, Goldstein, Matta, and Ratcliffe (1989). The peptides identified contained in these two proteins were grouped in families (Table 2, Fig. 3). There were

identified 9 families in 11S globulin and 6 families in agglutinin, corresponding to the 24 and 21% of the respective sequences.

Sequences of the peptides that were able to cross Caco 2-TC7 monolayer were obtained as described previously (Table 2, Fig. 3).

The identified sequences contained in the samples were compared with antithrombotic peptide sequences derived from food proteins. BIOPEP (Minkiewicz et al., 2008) was used as the reference database in which, up to June 5th of 2015, 56 antithrombotic peptides were found. None of them presented matches with the sequences found.

As a first approximation to the action mechanism, and taking into consideration that the microplate method studies the thrombin inhibition produced by the peptides, a database analysis was performed and specific criteria were established to identify those peptides prone to interact with this enzyme.

The criteria used to select potentially active peptides considered the similarity between physiological substrate sequences of thrombin (Huntington, 2005) and our peptides. Thrombin is a serine protease that participates at the end of the coagulation cascade, also known as the common pathway. Regulation of thrombin is performed via a complex mechanism involving, besides the active site, two exosites called I and II. Whereas the active site shows preference for positively charged amino acids, both exosites prefer negatively charged amino acids. While hydrophobic contacts provide the majority of the binding energy for exosite I interactions, they contribute minimally to the overall binding energy for exosite II (Huntington, 2005).

Thrombin interacts preferably with substrata that contain positively charged amino acids in a defined position, called P1 when Schechter and Berger (1967) nomenclature is used. Authors defined amino acids located in the cleavage zone of the peptide bond: H_2N-P4-P3-P2-P1–P1′-P2′-P3′-P4′-COOH. The protease cleavage site is indicated with two bold dashes (Huntington, 2005). Even though thrombin presents a complex recognition mechanism, it has been described that the enzyme presents preferences for sequences containing arginine in P1 position, proline in P2 and a hydrophobic residue, which usually is an aromatic amino acid, in P4 (Huntington, 2005; Mehta, Jin, & Desai, 2013). However, according to MEROPS database information, thrombin specificity (Rawlings et al., 2014) differs to some extent, as in position P4 thrombin interacts preferably with alanine, an aliphatic hydrophobic amino acid.

Parameters that involve characteristics and relative quantities of the amino acids were calculated according to Huntington (2005): *net negative charge fraction* (f_n) and *net hydrophobic fraction* (f_h). f_n is obtained by subtracting the basic from acidic residues, and dividing by the number of all residues, whereas f_h is the ratio of hydrophobic residues divided by the number of all residues. These parameters allowed us to estimate:

Probable interaction of the peptide with active site of the thrombin: the number of positively charged amino acids and their distribution in the sequences was analysed.

Probable interaction of the peptide with exosite I: when f_n/f_h results <2, there is high probability of interaction between the sequence and exosite I. Hydrophobic contacts provide the majority of the binding energy for exosite I interactions,

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Table 2 – Sequences identified in the active fraction and basolateral chamber of the inserts, by MALDI-TOF, LC–MS/MS, and MASCOT 2.2-Uniprot.

Protein reported in 2.2-Uniprot	Sequences found in AF[a]	Families
11S globulin (*A. hypochondriacus*)	32	9
Agglutinin (*A. caudatus*)	11	6
Pollen allergen (*A. retroflexus*)	8	5
Cystatin (*A. hypochondriacus*)	2	2
NAD-dependent malic enzyme (*A. hypochondriacus*)	2	2
Betaine aldehyde dehydrogenase (*A. hypochondriacus*)	1	1
NADH dehydrogenase (*A. blitoides*)	1	1
	Sequences found in b-ch[b]	
11S globulin (*A. hypochondriacus*)	2	2
Agglutinin (*A. caudatus*)	2	2
ATP synthase (*A. hypochondriacus*)	2	2
Malic enzyme (*A. hypochondriacus*)	1	1
Trypsin inhibitor (*A. hypochondriacus*)	1	1
Soluble starch synthase I (*A. hypochondriacus*)	1	1

[a] AF: active fraction.
[b] b-ch: basolateral chamber of the inserts.

I

II
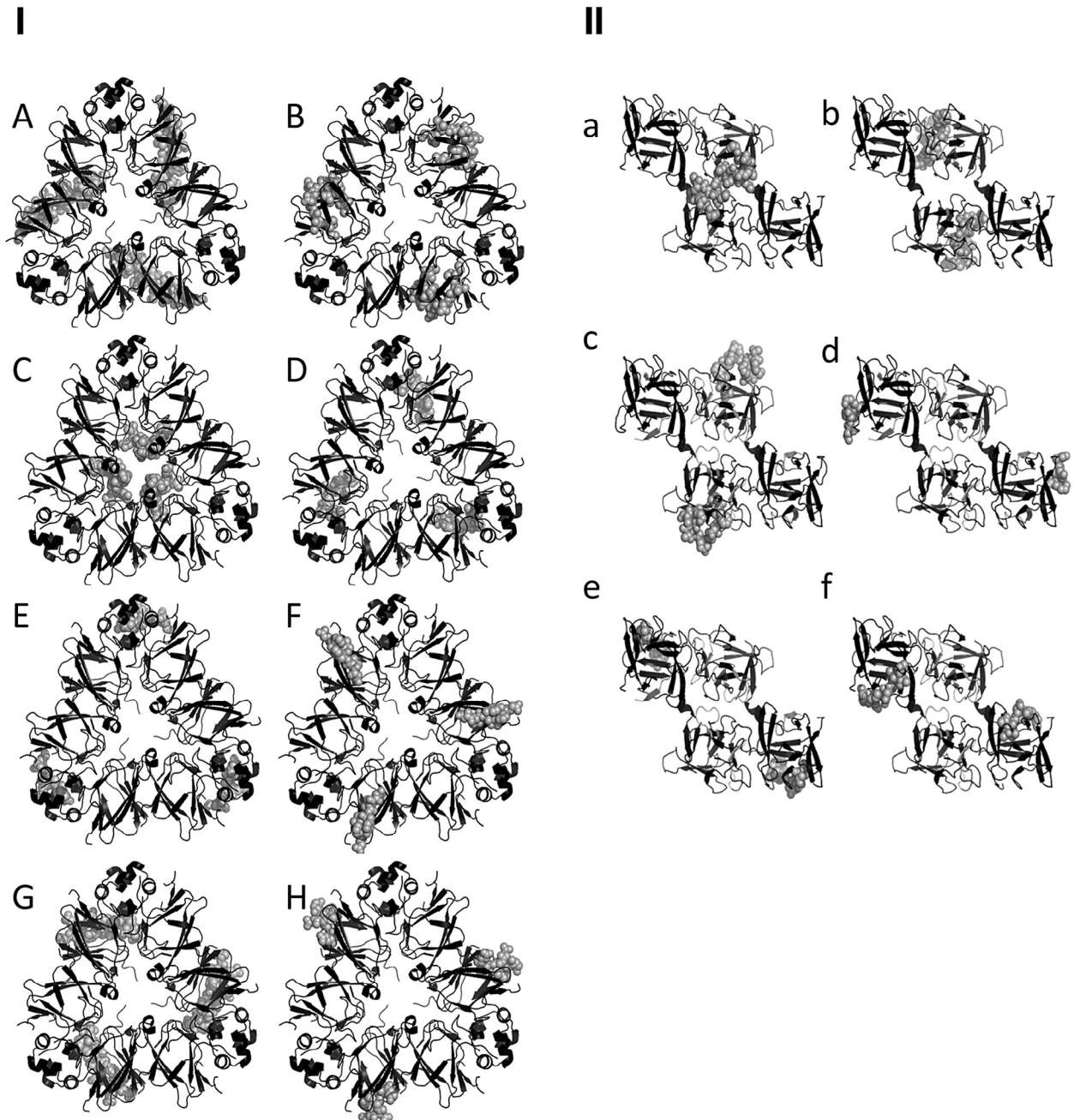
MEGRFREFQQGNECQIDRLTALEPTNRIQAERGLTEVWDSNEQEFRCAGV
SVIRRTIEPHGLLLPSFTSAPELIYIEQGNGITGMMIPGCPETYESGSQQ
FQGGEDERIREQGSRKFGMRGDRFQDQHQKIRHLREGDIFAMPAGVSHWA
YNNGDQPLVAVILIDTANHANQLDKNFPTRFYLAGKPQQEHSGEHQFSRE
SRRGERNTGNIFRGFETRLLAESFGVSEEIAQKLQAEQDDRGNIVRVQEG
LHVIKPPSRAWEEREQGSRGSRYLPNGVEETICSARLAVNVDDPSKADVY
TPEAGRLTTVNSFNLPILRHLRLSAAKGVLYRNAMMAPHYNLNAHNIMYC
VRGRGRIQIVNDQGQSVFDEELSRGQLVVVPQNFAIVKQAFEDGFEWVSF
KTSENAMFQSLAGRTSAIRSLPIDVVSNIYQISREEAFGLKFNRPETTLF
RSSGQGEYRRKISIA

XAGLPVIMCLKSNNHQKYLRYQSDNIQQYGLLQFSADKILDPLAQFEVEP
SKTYDGLVHIKSRYTNKYLVRWSPNHYWITASANEPDENKSNWACTLFKP
LYVEEGNMKKVRLLHVQLGHYTQNYTVGGSFVSYLFAESSQIDTGSKDVF
HVIDWKSIFQFPKGYVTFKGNNGKYLGVITINQLPCLQFGYDNLNDPKVA
HQMFVTSNGTICIKSNYMNKFWRLSTDDWILVDGNDPRETNEAAALFRSD
VIIDFNVISLLNMQKTWFIKRFTSGKPGFINCMNAATQNVDETAILEIIEL
GQNN

Fig. 3 – Sequences of potential thrombin inhibitors located in the protein structures and complete sequences of I **11S globulin homotrimer,** A. hypochondriacus **(UniProt:Q38712) and** II **agglutinin,** Amaranthus caudatus **(UniProt:Q71QF2). Grey rectangles indicate families of peptides identified in the active fraction and empty black rectangles show peptides identified in the basolateral chamber. 11S globulin structure is shown in standard orientation (IE side frontwards). The cleavage site where 11S globulin acidic and basic chains are linked is indicated in black.**

Table 3 – Sequences of potentially thrombin inhibitory peptides.

Family	Sequence	Probable thrombin site of interaction				Number of amino acids in the sequence
11S Globulin						
		f_n^b	f_h^b	f_n/f_h^b		
A	IQAEAGLTEV	0.2	0.2	1	Exosite I	10
A	TEVWDSNEQE	0.4	0.2	2	Exosite II	10
C	IDTANHANQLD	0.09	0.09	1	Exosite I	11
H	AFEDGFE	0.43	0.29	1.5	Exosite I	7
G	NDQGQSVFDEELS	0.31	0.15	2	Exosite II	13
C	IDTANHANQLDK	Active site				12
D	LAGKPQQEHSGEHQ	Active site				15
F	DVYTPE	b-ch[a]				6
Agglutinin						
d	DNLNDPK	Active site				7
e	LVDGNDPR	Active site				8
c	AESSQIDTGSK	b-ch[a]				11

[a] b-ch: peptides found in basolateral chamber of inserts.
[b] fn, fh negative and hydrophobic fraction by Huntington (2005).

with electrostatics mostly involved in orienting the complementary hydrophobic surfaces (Myles, Le Bonniec, Betz, & Stone, 2001).

Probable interaction of the peptide with exosite II: when f_n/f_h results ≥2, there is high probability of interaction between the sequence and exosite II. Electrostatic interactions are dominant when the peptides establish interactions with this exosite, while hydrophobic ones contribute minimally to the overall binding energy.

Based on the above criteria, sequences presented in Table 3 were identified as potential thrombin inhibitors. Peptides found in the basolateral chamber that were also found in AF were added to this list (Table 3) as clotting inhibition was observed when studying this sample. The peptides exhibited will be synthesized to confirm their bioactivity.

After performing an *in silico* simulation of the proteolysis employed with ExPASy peptide cutter tool (Gasteiger et al., 2005) of 11S globulin and agglutinin molecules, many cleavage sites that released the peptides identified were observed (results not shown). Pepsin (EC 3.4.23.1), trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) were considered as the main proteases involved in this process. Even though there were numerous cleavage sites in the sequences of the proteins that were revealed, the actual proteolytic attack will depend also in the location of these cleavage sites in the quaternary structure of the molecules. Fig. 3 presents the peptides shown in Table 3 located in 11S globulin and agglutinin structures. Most of the peptides are positioned in the surface of the molecules, being more exposed to possible proteolytic attacks. In this regard, *DVYTPE* peptide, found in the basolateral chamber, is located in the IE side of the 11S globulin trimer. In the native hexameric molecule, the IE side is bound by non-covalent interactions to the other homotrimer; hence, when the molecule is in its native form, with intact quaternary structure, this peptide would not be exposed (Tandang-Silvas et al., 2012). However, 11S globulin separates by pH effect ensuring accessibility of proteases to that region of the molecule.

4. Conclusions

The amaranth protein isolate obtained did not exhibit clotting inhibition at the concentrations studied. However, the enzymatic treatments performed simulating gastrointestinal digestion were able to generate peptides with potential antithrombotic activity, indicating that this methodology releases bioactive peptides which are encrypted in their native proteins. A fraction with high antithrombotic activity *in vitro* was obtained from this hydrolysate by size-exclusion chromatography, and resulted three times more potent than its original sample when analysed with the microplate method. The active protein fraction was sequenced and potentially bioactive peptides were found, in particular thrombin inhibitors, one of the possible mechanisms of action that is reflected in the microplate assay. The absorption *in vitro* assay through intestinal epithelium performed with the active fraction showed that some peptides are able to cross the Caco2-TC7 cell monolayer. After selecting the potentially bioactive peptides, it was observed that most of them are located in the surface of the molecules, being more exposed to proteolytic attacks. Thereby, it must be taken into consideration that an amaranth isolate could be used as a potential functional ingredient with antithrombotic peptides released after ingestion.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2015.10.015.

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