LWT - Food Science and Technology 60 (2015) 171–177

Contents lists available at ScienceDirect

LWT - Food Science and Technology

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

# Potential antithrombotic activity detected in amaranth proteins and its hydrolysates

Ana Clara Sabbione [a, b], Adriana Scilingo [a, b, *], María Cristina Añón [a, b]

[a] Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), CCT, CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), Calle 47 y 116 – 1900, La Plata, Argentina

[b] Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Argentina

ARTICLE INFO

*Article history:*
Received 21 April 2014
Received in revised form
7 July 2014
Accepted 11 July 2014
Available online 19 July 2014

*Keywords:*
Amaranth proteins
Antithrombotic activity
Bioactive peptides

ABSTRACT

Amaranth protein isolate and fractions were obtained and subjected to proteolysis in order to evaluate its potential antithrombotic activity. The proteins were first hydrolyzed with alcalase (pH 10, 37 °C) and then with trypsin (pH 8, 37 °C). The samples were characterized physicochemically and antithrombotic activity was evaluated using clotting tests (PT, TT and APTT) and the microplates assay. The fractions compared to the hydrolysates exhibited different electrophoretic profiles (tricine-SDSPAGE) and gel filtration chromatograms, evidencing the presence of different molecular species. The hydrolysis improved in every sample the bioactivity detected, excepting for the glutelin fraction, which exhibited the highest antithrombotic activity, significantly superior ($p < 0.05$) compared to the other fractions and the isolate. This behavior was observed in the two assays that analyzed the common path of the coagulation cascade at similar concentrations: TT (81.0 ± 8.5 s with a control of 19.5 ± 0.7 s) and microplate test ($IC_{50}$ 80 μg/mL), indicating a possible mechanism of action that involves the thrombin activity or the polymerization of fibrin monomers. The glutelin fraction showed a potential capacity to inhibit coagulation, appearing as a promising ingredient to formulate functional foods.

© 2014 Elsevier Ltd. All rights reserved.

## 1. Introduction

There is a relationship between food and many of the prevalent diseases in the world. A healthy diet combined with the intake of functional foods, defined as "those that contain a component that benefits a limited number of functions in the body, providing welfare and health, reducing the risk of disease" (Amer. Diet Assoc., 1999), could minimize the occurrence of certain diseases. This concept encourages us to explore biological properties of interest in multiple dietary sources, being the proteins from amaranth seeds an excellent choice, not only due to the higher concentration compared with that of cereals, but also because of its complete amino acid profile and high amount of lysine (Paredes Lopez, 1994). Several protein fractions are contained in amaranth seeds. Albumins, extracted from the flour with water (Martínez & Añón, 1996); globulins, soluble in saline solutions with pH near neutrality (Castellani, Martínez, & Añón, 2000); P-globulin, soluble in water and low ionic strength saline solutions; glutelins, soluble in acid or alkaline solutions (Abugoch, 2006).

There have been several reports of biological activities of peptides freed from dietary proteins. Some act on cardiovascular system, exhibiting antihypertensive, antioxidant, antithrombotic or hypocholesterolemic activity. Specifically, in amaranth were informed bioactive peptides exhibiting antitumoral (Barrio & Añón, 2010), antihypertensive (Vecchi & Añón, 2009), antioxidant (Orsini Delgado, Tironi & Añón, 2011) and hypocholesterolemic activities (Mendonça, Saldiva, Cruz & Arêas, 2009). Antithrombotic activity of amaranth proteins and peptides is an unexplored field yet to be informed.

The aim of this research was to analyze the antithrombotic activity from amaranth proteins or protein hydrolysates, in order to generate knowledge that we could use as a ground for the developing of new biologically active ingredients from this pseudocereal. Our purpose is to obtain protein fractions and its hydrolysates, to characterize them structurally and physicochemically, and to evaluate its antithrombotic activity using global tests of hemostasis and *in vitro* microplate assay (Yang, Wang, & Xu, 2007).

* Corresponding author. CIDCA, calle 47 y 116 – 1900, La Plata, Argentina. Tel.: +54 221 4249287; fax: +54 221 4254853.

*E-mail addresses:* acsabbione@gmail.com, anaclara_s9@hotmail.com (A.C. Sabbione), ascilingo@quimica.unlp.edu.ar (A. Scilingo), mca@biol.unlp.edu.ar (M.C. Añón).

http://dx.doi.org/10.1016/j.lwt.2014.07.015
0023-6438/© 2014 Elsevier Ltd. All rights reserved.

## 2. Materials and methods

### 2.1. Plant material and samples preparation

#### 2.1.1. Amaranth seeds

*Amaranthus mantegazzianus* (Pass cv Don Juan) seeds harvested at Estación Experimental Agropecuaria del INTA, Anguil, La Pampa, Argentina were used.

#### 2.1.2. Amaranth flour preparation

Seeds were ground and sieved through a 0.092 mm mesh at Facultad de Ciencias Agrarias y Forestales, UNLP. The resulting flour was defatted with n-hexane from Anedra (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 isolates were obtained from the defatted flour by extraction at pH 9, isoelectric precipitation (pH = 5), neutralization and lyophilization (Martínez & Añón, 1996).

#### 2.1.4. Preparation of protein fractions

Amaranth protein fractions were obtained from defatted flour as described by Martínez and Añón (1996).

#### 2.1.5. Preparation of hydrolysates

Isolate and protein fractions were resuspended in distilled water (10 g sample/100 mL), pH was adjusted to 10 (1 h stirring, 37 °C) and alcalase (2.4 L Sigma Chemical Co., microbial protease of *Bacillus licheniformis*, specific activity 2.4 Anson units/g) was added, 0.08 μL enzyme solution/mg of sample, and incubated (20 min stirring, 37 °C). Then the trypsin of bovine pancreas (Sigma Chemical Co., 0.05 BTEE units/mg solid), 0.01 mg enzyme/mg sample was added (20 min stirring, 37 °C). The proteolysis was stopped by heating (85 °C, 10 min) and the hydrolysate was lyophilized. Aliquots were taken at different reaction times. The time necessary to reach the temperature that produces the inactivation of proteases was called zero time (0 min).

### 2.2. Composition of samples

#### 2.2.1. Total protein content

Total protein content was obtained by using the micro-Kjeldahl method. The ammonium released was quantified with the colorimetric Nkonge and Balance method (Nkonge & Ballance, 1982). Conversion factor 5.85 g protein/g nitrogen (Paredes-López, 1994). Determinations were performed in duplicate for all samples.

#### 2.2.2. Soluble protein

Soluble protein prior to studying the bioactivity, was determined using Lowry colorimetric method (Lowry, Rosebrough, Farr, & Randall, 1951).

#### 2.2.3. Ash percentage

A known amount of sample was placed in a porcelain capsule; it was carbonized and incinerated in a muffle furnace at 550 °C, until white ashes. Once cool, the capsule was weighed and the ash percentage was calculated.

#### 2.2.4. Carbohydrates content

Carbohydrate content was determined by the anthrone method (Yemm & Willis, 1954). The sample was previously subjected to acid hydrolysis with hydrochloric acid at boiling under reflux for two hours, in order to achieve a homogeneous sample.

### 2.3. Degree of hydrolysis

Free amino groups were quantified by using the trinitrobenzenesulfonic acid (TNBS, Sigma Chemical Co.) described by Adler-Nissen (1979). To calculate the degree of hydrolysis the following expression was used:

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

$NH_{2\ t}$, $NH_{2\ t0}$ 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 a sample of isolated non-hydrolyzed, and $NH_{2\ t\infty}$ was obtained to the following expression:

$$NH_{2\ t\infty} = (1/MW_{aa}) \times \left(1 + f_{Lys}\right) \times [P] \times 1000 \quad (2)$$

where $MW_{aa}$ is the average molecular weight of the amino acids of amaranth proteins, 130 g/mol, $f_{Lys}$ is lysine proportion in amaranth proteins, 1/16 and [P] is isolate protein concentration (Bressani, 1994). Free amino groups of the isolate and native fractions with and without 10 min heating at 85 °C were measured as a control.

### 2.4. Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine SDS-PAGE)

Soluble fractions were analyzed by tricine-SDS-PAGE (Schägger, 2006). Runs were carried out in stacking, spacing and separating gels using 160, 100 and 40 g/L acrylamide respectively. For runs under reducing conditions the sample buffer contained 50 mL/L of 2-mercaptoethanol (2-ME). Two protein molecular mass standards were used: phosphorylase-b (94 kDa); bovine serum albumin (67 kDa); ovalbumin (43 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (20.1 kDa); α-lactalbumin (14.4 kDa) from GE Healthcare, and triose-phosphate isomerase (26.6 kDa); myoglobin (16.95 kDa); α-lactalbumin (14.43 kDa); aprotinin (6.51 kDa) from Bio-Rad. Gels were fixed in a methanol-acetic acid solution and stained with Coomassie Brilliant Blue R-250 (Anedra). In some cases silver staining was applied to increase analytical sensitivity. Gels images were analyzed with an image processing program (Image J). Electrophoretic runs were repeated twice.

### 2.5. Molecular exclusion chromatography

Soluble fractions were analyzed in a Pharmacia LKB, FPLC System, using two different molecular exclusion columns (GE-Healthcare total volume, Vt = 25 mL), Superdex-75 10/300-GL column (range: 3–70 kDa) and Superose-6 N°3 (range: 5–5000 kDa). The first one was calibrated with blue dextran (exclusion volume, Vo = 7.3 mL), albumin (67 kDa), ovoalbumin (44 kDa), chymotrypsin (25 kDa), ribonuclease (19 kDa) and aprotinin (6.5 kDa) from GE Healthcare, obtaining the following calibration equation:

$$\log MW = -0.1104\ Ve + 2.701 \quad (3)$$

MW is molecular weight and Ve is the elution volume of the resolved species.

The column Superose 6 N°3 was calibrated with blue dextran (exclusion volume, Vo = 7.16 mL), thyroglobulin (669 kDa), alcohol dehydrogenase (150 kDa), albumin (67 kDa), ovoalbumin (44 kDa), and ribonuclease (19 kDa) from GE Healthcare. The molecular masses of the fractions were calculated using the equation:

$$\log MW = -0.2526\ Ve + 5.976 \quad (4)$$

Samples were dissolved and eluted in three different buffers: amaranth isolate, globulin P and their hydrolysates in buffer 28 mmol/L $Na_2HPO_4$, 7 mmol/L $NaH_2PO_4$, pH 7.8; albumins, globulins and their hydrolysates in buffer 32.5 mmol/L $K_2HPO_4$, 2.6 mmol/L $KH_2PO_4$, 0.4 mol/L NaCl, pH 7.5; and glutelins and glutelins hydrolyzed in buffer 0.1 mol/L sodium borate. For both columns, 200 μL mL of soluble samples were loaded and eluted at 0.2 mL/min. Polypeptides and peptides were detected by absorbance at 280 nm. Every determination was performed at least twice.

### 2.6. Differential scanning calorimetry (DSC)

DSC measurements were performed in a TA-Q100 calorimeter (TA-Instruments, New Castle, DE). Hermetically sealed aluminum pans were prepared to contain 10–15 mg of sample suspended in water (20–40 mg sample/100 mL); a double empty pan was employed as reference. Capsules were heated from 20 to 140 °C at a rate of 10 °C/min. After each run pans were punctured and their dry-matter content was determined by leaving the pans overnight in an oven at 105 °C and weighted. Thermograms were analyzed with the Software Universal Analysis 2000 (version 4.4A. Software Plus V5.41). Every determination was performed at least twice.

### 2.7. Determination of antithrombotic activity

#### 2.7.1. Method of microplates (MM)

Antithrombotic activity was determined using the microplate method (Yang et al., 2007). The isolate, protein fractions and its hydrolysates were solubilized in the buffer of the assay (50 mmol/L Tris-HCl, pH 7.2, 0.12 mmol/L NaCl) to obtain a range of concentrations. In a microplate were added 140 μL of 0.1 g/100 mL fibrinogen (Sigma Chemical Co.), 40 μL of every sample or buffer as a negative control of the coagulation inhibition or 0.3 mg/mL heparin (Abbott) as a positive control of the coagulation inhibition. The coagulation process was initiated after adding 10 μL of thrombin from Sigma Chemical Co. (12 UI/mL). Absorbance at 405 nm was measured in a microplate reader (BioTek Synergy HT) before adding the enzyme and after the 10 min incubation at 37 °C. The percentage of inhibition (% Inhibition) was calculated with the following equation:

$$\%\text{Inhibition} = [(C - CB) - (S - SB)]/(C - CB) \times 100 \quad (5)$$

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.
Every determination was performed in triplicate.

#### 2.7.2. Clotting tests

*2.7.2.1. Human plasma preparation for clotting tests.* Plasma was collected from healthy individual donors and collocated into conical tubes with 2.5 g/100 mL sodium citrate solution from Wiener Lab (1:9 sodium citrate:blood). The plasma was separated from blood cells by centrifuging at 1600 × *g* during 15 min.

*2.7.2.2. Activated partial thromboplastin time (APTT) test.* Plasma (75 μL) was mixed with 25 μL from the supernatant obtained from dissolving each sample in the buffer of the assay or the same volume of buffer without sample, and was incubated for 1 min at 37 °C. The APTT reagent (100 μL) was added into the mixture and incubated for 3 min at 37 °C. The clotting time was determined after adding 100 μL of 25 mmol/L $CaCl_2$.

*2.7.2.3. Prothrombin time (PT) test.* Plasma and the PT reagent were preincubated during 2–3 min at 37 °C. Then 25 μL of the soluble fraction of samples in the buffer of the assay or the same volume of buffer without sample was added into 75 μL human plasma at 37 °C; quickly were added to the tube containing 200 μL of reagent PT and the clotting time was recorded.

*2.7.2.4. Thrombin time (TT) test.* 150 μL of plasma were mixed with 50 μL of the soluble fraction of each sample in the buffer of the assay or with the same volume of buffer. They were incubated 2 min at 37 °C and immediately the clotting time was determined after inducing the clotting by adding 200 μL of the TT reagent 2.3 NIH/mL.

Every determination of the clotting tests was performed at least twice using Wiener Lab kits.

### 2.8. Statistical analysis

Isolates and protein fractions were prepared several times (two at least). Results were evaluated statistically by using variance analysis (ANAVA). Comparison of means was performed to study the differences between the values obtained by using LSD Fisher test ($p = 0.05$).

## 3. Results and discussion

### 3.1. Samples characterization

In order to characterize the samples under study, protein content from amaranth isolate and protein fractions was determined. The values (Table 1) of protein isolate are similar to those previously informed by Martínez and Añón (1996) and Condés, Scilingo, and Añón (2009). While the protein content of albumins, globulins and P-globulin were similar (Alb, Glb and GP, Table 1), the protein content of the glutelins resulted significantly lower (Glt, Table 1). Isolate (Iso), Alb, Glb and GP contain, apart from proteins, water, salt and remnants of carbohydrates. Glt ash and carbohydrates content was determined, obtaining 17.9 ± 0.8 g/100 g solid and 43.30 ± 0.02 g/100 g solid respectively. During the successive steps of the fractions extraction, the starch, present in amaranth flour in small granules, remains insoluble until the alkaline pH used to obtain glutelins partially solubilizes it, leading to the co-extraction with the proteins (Han & Lim, 2004).

The amaranth isolate and the protein fractions underwent the hydrolysis of two enzymes, alcalase and trypsin successively. Fig. 1 shows as example the increasing of the hydrolysis degree (DH%) over time of amaranth isolate. The DH% of aliquots taken at different reaction times increased significantly ($p < 0.05$) over time since the beginning of the proteolysis. It is shown an abrupt increment of DH% when trypsin is added, increasing more slowly over the last 20 min of reaction. This was observed in all the samples and could be due to structural changes generated by alcalase that allow the exposition of numerous trypsin cleavage sites. As shown in Table 1, final products present a high hydrolysis degree after 40 min reaction. GP turns out more resistant to the hydrolysis, whereas Alb and Glb presented similar DH%. Glt and Iso ($p < 0.05$) expressed the highest values. Possibly, the high polymerization degree of GP (Castellani et al., 2000) explains why it is difficult for enzymes to reach the specific cleavage sites and the fact

**Table 1**
Protein content, thermal parameters and degree of hydrolysis of amaranth samples.

| | | Sample | | | | |
|---|---|---|---|---|---|---|
| | | Protein isolate | Protein fraction | | | |
| | | | Albumins | Globulins | Globulin-P | Glutelins |
| Abbreviation | | Iso | Alb | Glb | GP | Glt |
| Protein (g/100 g) | | 73.8 ± 1.0[b] | 73.2 ± 2.4[b] | 72.6 ± 1.3[b] | 79.1 ± 1.8[c] | 32.1 ± 1.8[a] |
| Thermal parameters | Td (°C) | 70.8* | nd | 72.4 ± 0.2 | 103.9 ± 0.5 | nd |
| | | 100.5 ± 1.6 | | 97.5 ± 0.1 | | |
| | $\Delta H$ (J/g) | 9.0 ± 0.9 | nd | 11.7 ± 1.1 | 7.5 ± 0.6 | nd |
| Degree of hydrolysis (DH%) | | 25.8 ± 2.2[c] | 15.7 ± 0.5[b] | 14.2 ± 0.5[b] | 10.6 ± 0.1[a] | 25.9 ± 0.1[c] |

nd: not detected.
Different superscript letter in a same line corresponded at different values ($p < 0.05$).
* It was detected in only one thermogram.

that Glt require alkaline pH conditions for its extraction generating lost of conformational structure and hence, a more unfolded protein, could explain the reason why this fraction exhibited the highest DH%.

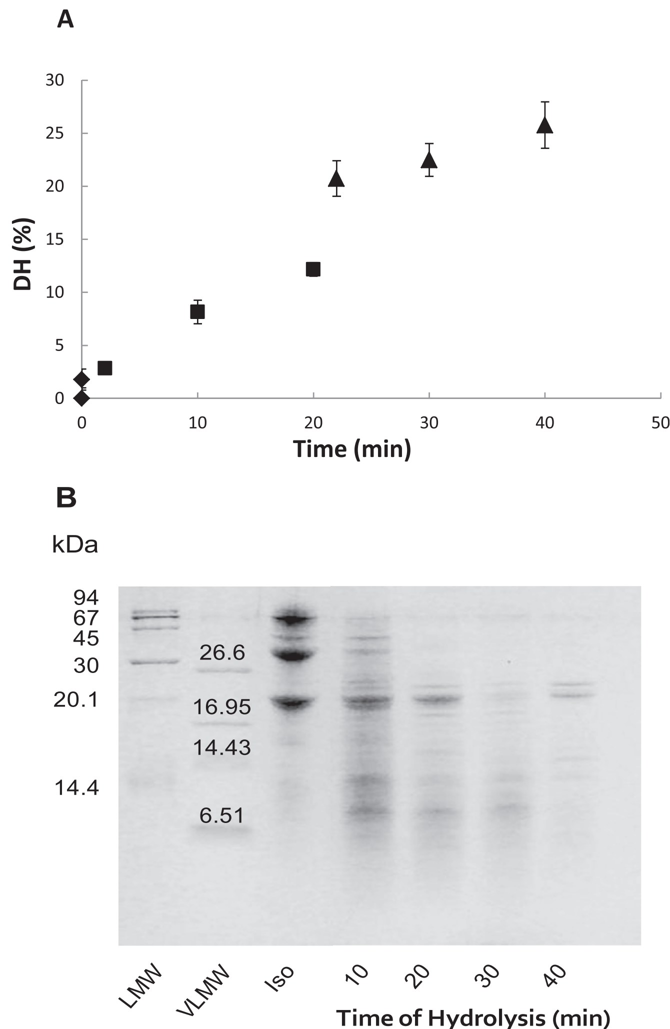


**Fig. 1.** Hydrolysis evolution with time. A: Degree of Hydrolysis (DH) of amaranth isolate. Control (rhombus), Alcalase (squares) and Trypsine + Alcalase (triangles). Error bars refer to standard deviation. B: Tricine-SDS-PAGE in presence of 2-ME. Amaranth isolate (Iso) and hydrolyzed at different times (0, 10 and 20 min, with Alcalase, and 30 and 40 min with Trypsine + Alcalase). LWM: standards low molecular weight. VLMW: standards very low molecular weight.

Fig. 1B shows electrophoretic profiles obtained in tricine gel with Iso and IsoH at different proteolysis times. Iso, consisting mainly in globulins, presents numerous characteristic bands of its constitutive polypeptides: high molecular mass aggregates, molecular units of approximately 54–56 kDa, and polypeptides nearby 30 and 20 kDa, acidic and basic subunits, that are linked by disulfide bonds in globulin 11S and P (Martínez & Añón, 1996; Segura-Nieto, Barba de la Rosa, & Paredes-López, 1994). There are also bands of 67 kDa and of low molecular mass that correspond with polypeptides of globulin 7S, present in a minor quantity in amaranth as described by Quiroga, Martínez, Rogniaux, Geairon, and Añón (2010). When they are subjected to hydrolysis, the electrophoretic profile gets modified. A large increase of the number and intensity of molecular mass bands inferior to 26 kDa is observed even at short reaction times. As time increases, the profile gets fainter, indicating that those polypeptides are the substrata of the enzymes and turn into smaller peptides.

Fig. 2 exhibits electrophoretic profiles of isolate, protein fractions and its hydrolysates obtained in complete reaction time. Fraction profiles show polypeptides already described in previous works (Abugoch, 2006; Castellani et al., 2000; Martínez & Añón, 1996). Even though Alb fraction presents a high quantity of low molecular weight polypeptides (inferior to 14.4 kDa), the other fractions show an increase of them only after being subjected to the proteolysis reaction. All hydrolysates exhibit significant modifications in the profiles compared to the fractions without proteolysis. It is observed, particularly, the disappearance of high molecular mass bands and the appearance of peptides that present molecular weights inferior to 14.4 kDa. The AlbH fraction contains several low molecular weight peptides that are not able to define themselves as bands but appear as a continuous. Although there are several differences in the polypeptides proportion that conform Glb and GP, this fractions present similar polypeptide composition, as described by Castellani et al. (2000). Once subjected to hydrolysis, significant changes are observed in these samples. Both profiles (GlbH and GPH) exhibit the same bands but a difference in intensity is detected. However, the most intense band is the same in both samples and could possibly be constituted by more than one polypeptide, whose molecular mass would be close to each other (23 kDa approximately). The similitude of the hydrolyzed profiles confirms that Glb and GP are similar substrata for the proteases used. The Glt fraction also presented similarities with Glb and GP profiles, but once hydrolyzed its profile showed three bands of molecular mass nearby 6.51 kDa well defined. The GlbH and GPH fractions show polypeptides between 20 and 26 kDa as the most intensive bands and present a set of peptides that corresponds to lighter bands.

The denaturation temperatures (Td) and enthalpies ($\Delta H$) obtained from the thermograms of the samples are shown in Table 1.

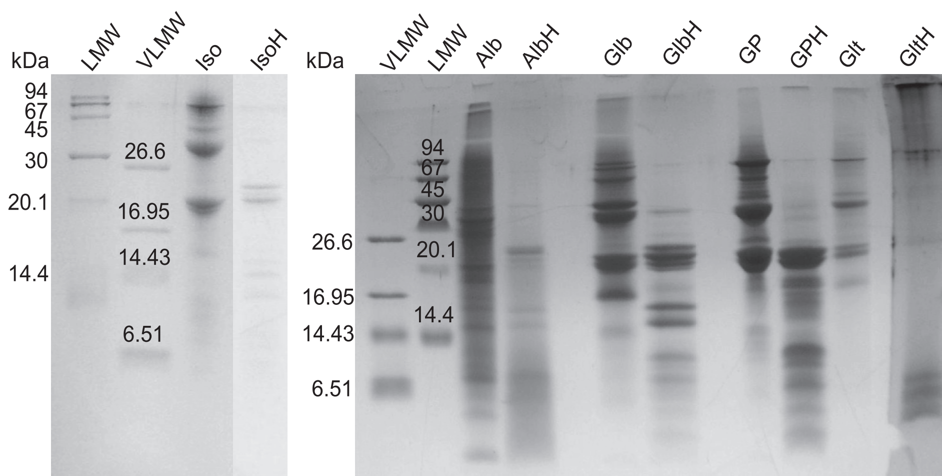


**Fig. 2.** Tricine-SDS-PAGE in presence of 2-ME. Iso, Alb, Glb, GP and Glt without or with hydrolysis. LWM: standards low molecular weigth. VLMW: standards very low molecular weight.

The isolate exhibited an endotherm (Td = 100.5 ± 1.6 °C) with a denaturation enthalpy ($\Delta H$) of 9.0 ± 0.9 J/g. This $\Delta H$ was similar to what described Martínez and Añón (1996). The endotherm corresponds to the highest thermal stability peak, in which globulin and P-globulin are denatured. As described by Martínez and Añón (1996) and Condés et al. (2009) it is observed an endotherm at lower temperatures (Td = 70.8 °C approximately), associated to the denaturation of other protein fractions present with globulins, or to the presence of albumins. However, this endotherm was detected only in one thermogram and its enthalpy resulted lower than the expected, inferior to 1.0 J/g. This result would indicate that the quantity of albumins in the isolate is very small, or that it could have suffered denaturation in some stage of the seeds manipulation. The latter hypothesis gets reinforced because albumins did not show any endotherm associated to its denaturation (Table 1). Tang & Wang (2010) did not find any distinct transition for albumins fraction of the backwheat. They proposed that as the endothermic peak in the DSC-thermograms of proteins is usually related to disruption of hydrogen bonds, especially those maintaining the integrity of tertiary structure of globulins; the absence of endothermic peaks may be due to weak hydrogen bond interactions that maintain the tertiary conformation, or to a high flexibility in tertiary conformation of the albumin polypeptides. Other authors (Martínez & Añón, 1996) informed thermal transitions of amaranth albumins, presenting low Td and $\Delta H$ (approximately 2.0 J/g). The Glb and GP fractions presented similar values of temperature and denaturation enthalpy (Table 1) to those informed by Martínez and Añón (1996). Glt did not show any endotherms associated to the warming process performed in the DSC. As glutelins require alkaline pH conditions for its extraction, it is possible that during this process they lost their native structure. The DSC analysis of hydrolyzed samples showed the total absence of endotherms. The results indicate that structural changes occur that generate the lost of native conformation detected in Iso, Glb and GP, fractions that initially presented a denaturation peak (Table 1). This could be adjudge to the proteolysis, even though the effect of pH and temperature conditions used during the hydrolysis cannot be discarded.

The effect of hydrolysis over amaranth proteins (IsoH or fractionsH) is observed in gel filtration chromatograms obtained by using FPLC system (Fig. 3). Iso, Glb, GP and Glt and their hydrolysates were separated in Superose 6 column (5–5000 kDa), while Alb and AlbH were separated in Superdex 75 (3–70 kDa). Iso presents a similar profile to one previously described by Martínez, Castellani, and Añón (1997). Species with molecular masses over 100 kDa, among them P-globulin and 11S of 280–300 kDa, and globulin 7S, of 180 kDa (Quiroga et al., 2010) lie on the prime zone of the chromatogram (elution volume to 15.5 mL approximately), whereas species of molecular masses below 100 kDa correspond mainly to albumins and are located in higher volumes. IsoH does not present peaks in the zone of high molecular mass, while the area of the peak in the latter zone of the chromatogram increases

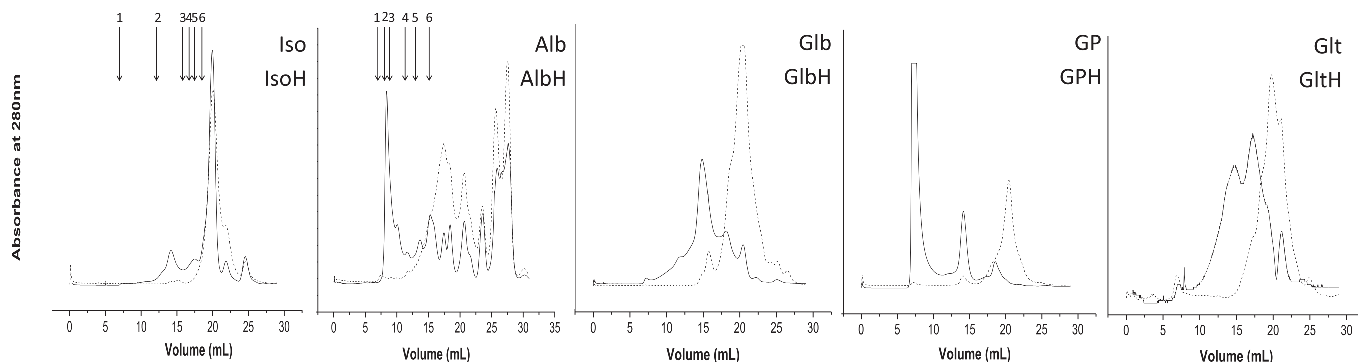


**Fig. 3.** FPLC chromatograms of amaranth isolate (solid line), protein fractions (solid line) and their hydrolysates (dotted lines). Iso, IsoH, Glb, GlbH, GP, GPH, Glt, and GltH were separated on Superose 6 (standard calibration: thyroglobulin (2), alcohol dehydrogenase (3), albumin (4), ovoalbumin (5), ribonuclease (6)). Alb and AlbH, using Superdex 75 (standard calibration: albumin (2), ovoalbumin (3), chymotrypsin (4), ribonuclease (5), aprotinin (6)). Exclusion volume: blue dextran (1).

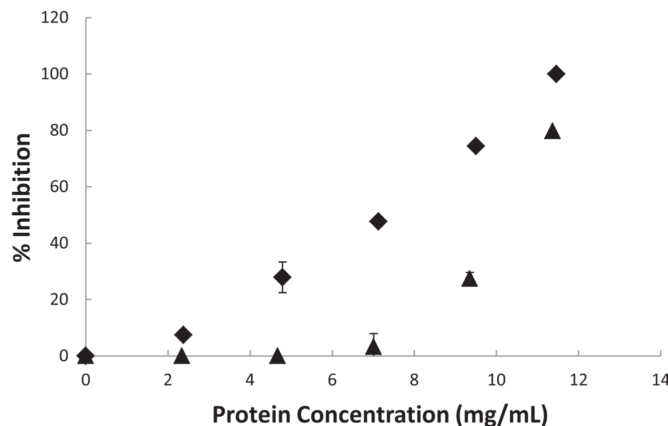


**Fig. 4.** Percentage of inhibition of thrombus formation vs protein concentration. Amaranth albumin (triangles) and amaranth albumin hydrolysate (rhombus). Error bars refer to standard deviation.

and slightly moves to higher volumes. This behavior is also observed in the chromatograms of the hydrolyzed fractions compared to those without enzymatic treatment. The proteolysis generates the appearance of lower molecular mass species that elute at higher volumes.

### 3.2. *Antithrombotic activity in vitro*

The potential antithrombotic activity of proteins and protein hydrolysates was evaluated with the microplates assay, in which ability to prevent formation of the fibrin clot is evaluated by looking at the diminution or total absence of turbidity. The assay emulates the last stage of blood coagulation, where thrombin proteolyzes the fibrinogen to generate fibrin monomers that subsequently polymerize. The inhibitory action of proteins and protein hydrolysates may be due to the union to one or both of the active sites of thrombin, preventing the proteolysis of the fibrinogen, or to its binding to already generated fibrin monomers preventing its polymerization (Laudano & Doolittle, 1978). Fig. 4 shows the curve %Inhibition vs protein concentration of Alb and AlbH as an example. These data were processed with the GraphPad Prism program to calculate the $IC_{50}$ of each sample (concentration that inhibits the 50% of the thrombus formation) from the inhibition curves obtained (Table 2). Hydrolysis seems to increase the potential antithrombotic activity of Iso, Alb and Glb, with the exception of Glt that resulted more effective than GltH. The antithrombotic activity of GP could not be determined as it is not able to solubilize in the buffer of the assay, generating thereby an incompatible turbidity with the spectrophotometric reading on which the method relies.

Results indicate that Glt and GltH present the highest antithrombotic activity because concentrations in the order of μg/mL inhibit 50% of coagulation. These values are significantly inferior to those informed by Zhang, Wang, and Xu (2008), who found 30 mg/mL in rapeseed peptides, and by Yang et al. (2007), who informed $IC_{50}$ above 50 mg/mL in egg white proteins and its hydrolysates. Results support the effectiveness of our samples in the inhibition of clotting, presenting potential antithrombotic activity, increasing its effect, all except Glt, when the proteolytic treatment is performed. It was previously described that Glt present high salt concentration (17.9 ± 0.8 g/100 g of glutelins). In order to adjudge the biological activity to proteins and discard possible effects that could alter the clot formation due to the presence of salts, a control assay was performed with different NaCl concentrations (0–20 mg/mL). It was observed that at high NaCl concentrations, the clot generated does not exhibit turbidity, leading to false results, but these ionic strength values are above those contained in Glt, confirming the bioactivity of the proteins.

In addition to the microplate assay, clotting tests (WienerLab kits), commonly used in clinical biochemistry, were performed (Table 3). These tests evaluate potential antithrombotic activity of amaranth proteins and protein hydrolysates in different stages of the coagulation cascade. Prothrombin Time (PT) evaluates the extrinsic pathway of coagulation. The samples studied in the concentrations specified in Table 3 did not induce changes in clotting times of PT compared to control plasma, with the exception of Glt, that was able to delay the clot appearance for more than 6 s with a very low concentration in plasma (Glt, Table 3, $p < 0.05$).

Thrombin Time (TT) evaluates the conversion of fibrinogen in fibrin through the action of thrombin, thereby presence of substances that interfere with the action of the enzyme over fibrinogen or block polymerization of fibrin monomers lead to its prolongation. The results obtained show that all hydrolyzed samples produced a delay in TT. Except for Glt, which was the most active fraction, even more than its hydrolysate, the proteolysis was a valuable tool that enhanced bioactivity in amaranth proteins. Therefore, Glt generates the maximum delay of clot formation (60 s) with the lowest concentration in plasma (50 μg/mL) followed by GltH that produces a 10 s delay at a concentration nearby 500 μg/mL.

Results of this test correlated with those obtained in the microplate assay. The two methods analyze the same pathway of the coagulation process and the concentrations in which the samples showed bioactivity were similar.

APTT is a sensitive test to detect abnormalities in the intrinsic pathway. Exhibits deficiency of procoagulant factors in plasma and presence of certain coagulation inhibitors. Unlike TT and TP tests, the results obtained from APTT indicate that Glt did not exhibit any effect over the intrinsic pathway, whereas albumins were capable of delaying thrombus formation for over 40 s with a concentration in plasma below 4 mg/mL. These results do not contradict with the other effects previously mentioned, as the APTT studies different coagulation aspects than those analyzed in the other clotting tests. Thereby, different protein fractions produce the maximum inhibitory effect in distinct screening tests. In the APTT assay the non-hydrolyzed protein fractions presented a higher delay compared to those that suffered the proteolytic treatment, while in the other tests, except for Glt, the hydrolysates showed a higher antithrombotic activity.

Samples that produced significant coagulation delays in TT and APTT were tested at lower concentrations than those exhibited in

**Table 2**
The concentration of protein samples and their hydrolysates, that inhibits the 50% of clot formation.

| | Sample | | | | |
|---|---|---|---|---|---|
| | Iso | Alb | Glb | GP | Glt |
| $IC_{50}$ mg/mL protein sample | nd | $10.160 \pm 0.001^{de}$ | nd | – | $0.080 \pm 0.003^{a}$ |
| $IC_{50}$ mg/mL Protein sample hydrolysates | $10.87 \pm 1.00^{e}$ | $7.36 \pm 0.12^{c}$ | $9.379 \pm 0.001^{d}$ | nd | $0.74 \pm 0.07^{b}$ |

nd: $IC_{50}$ no determined at used concentrations.
Different superscript letter corresponded at different values ($p < 0.05$).

**Table 3**
Clotting tests: Prothrombin time (PT) test, Thrombin time (TT) test, and Activated partial thromboplastin time (APTT) test.

| Sample | PT (s) | Protein concentration (mg/mL) | TT (s) | Protein concentration (mg/mL) | APTT (s) | Protein concentration (mg/mL) |
|---|---|---|---|---|---|---|
| Human control plasma | $14 \pm 0.0^{a}$ | 0.0 | 19.5 ± 0.7 ab | 0.0 | 37.0 ± 1.4a | 0.0 |
| Iso | $15.5 \pm 0.7^{ab}$ | 1.8 ± 0.2 | $21.0 \pm 0.0^{ab}$ | 2.7 ± 0.2 | $59.5 \pm 0.7^{e}$ | 1.8 ± 0.2 |
| IsoH | $18.5 \pm 0.7^{c}$ | 5.4 ± 0.5 | $39.5 \pm 0.7^{f}$ | 8.1 ± 0.8 | $52.0 \pm 2.8^{cd}$ | 5.4 ± 0.5 |
| Alb | $18.0 \pm 0.0^{c}$ | 3.7 ± 0.1 | $25.0 \pm 0.0^{bcd}$ | 5.6 ± 0.2 | $82.8 \pm 2.8^{f}$ | 3.7 ± 0.1 |
| AlbH | $17.5 \pm 0.7^{c}$ | 3.76 ± 0.01 | $33.0 \pm 0.0^{de}$ | 5.64 ± 0.05 | $53.0 \pm 1.4^{d}$ | 3.76 ± 0.01 |
| Glb | $14.5 \pm 0.7^{a}$ | 0.54 ± 0.02 | $20.0 \pm 0.0^{ab}$ | 0.81 ± 0.03 | $41.0 \pm 1.4^{b}$ | 0.54 ± 0.02 |
| GlbH | $17.0 \pm 0.0^{bc}$ | 2.48 ± 0.03 | $28.0 \pm 0.0^{cde}$ | 3.73 ± 0.05 | $39.5 \pm 0.7^{ab}$ | 2.48 ± 0.03 |
| GP | $15.0 \pm 0.0^{a}$ | 2.30 ± 0.04 | $18.5 \pm 0.7^{a}$ | 3.45 ± 0.6 | $52.0 \pm 2.8^{cd}$ | 2.30 ± 0.04 |
| GPH | $15.5 \pm 0.7^{ab}$ | 2.22 ± 0.03 | $23.5 \pm 0.7^{abcd}$ | 3.33 ± 0.04 | $42.5 \pm 0.7^{b}$ | 2.22 ± 0.03 |
| Glt | $21.5 \pm 2.1^{d}$ | 0.03 ± 0.01 | $81.0 \pm 8.5^{g}$ | 0.05 ± 0.01 | $49.0 \pm 1.4^{c}$ | 0.03 ± 0.01 |
| GltH | $15.5 \pm 0.7^{ab}$ | 0.35 ± 0.02 | $29.5 \pm 0.7^{d}$ | 0.52 ± 0.03 | $39.5 \pm 0.7^{ab}$ | 0.35 ± 0.02 |

Different superscript letter in a same column corresponded at different values ($p < 0.05$).

Table 3. Clotting times resulted similar or identical to controls, indicating dose-response behavior in all of them (results not shown).

Results obtained for TT test are comparable to those described by Jung and Kim (2009) that determined anticoagulant activity from marine bivalves. They found a prolonged blood clotting time on TT (11.6 ± 0.4 s to 42.1 ± 0.9 s) with 3.8 mg/mL of total protein soluble extract of *Mytilus edulis*, and 81.3 ± 0.8 s with the anticoagulant peptide purified (MEAP). Jung et al. (2007) also described an inhibitory protein purified from muscle of granulated ark (purified T. granosa anticoagulant protein, TGAP) that potently prolonged the normal clotting time on TT to 112.8 ± 1.2 s. Glt exhibited a similar bioactivity to MEAP and TGAP without any purification step, indicating the presence of potentially anticoagulant proteins and protein hydrolysates with higher activity in this amaranth fraction. Even though the fractions mentioned showed significant bioactivity, they are not comparable to heparin, a commercial anticoagulant that at 2.8 μg/mL was able to prolong for more than 300 s on APTT, PT and TT (Jung, Je, Kim, & Kim, 2001).

## 4. Conclusions

The enzymatic treatment of amaranth proteins used in this work, employing two proteases, is able to produce low mass and intermediate size molecules that present important structural differences compared to their native proteins. This proteolysis generally increases the potential antithrombotic activity, generating protein hydrolysates with higher bioactivity. Alb exhibits the maximum inhibition in the thrombus formation in APTT test while Glt and GltH present the maximum inhibition of the thrombus formation in MM and TT tests. In this paper it is shown for the first time the potential antithrombotic activity of amaranth proteins and protein hydrolysates, which is significantly higher than other food proteins. Our results indicate that the involved mechanism could be the blocking of the polymerization of fibrin monomers due to its binding to the fibrinogen or the inhibition of thrombin activity, both mechanisms related with the common path of coagulation.

## Acknowledgments

This work was supported by PIP 11220110101109. We also wish to thank Mariana M. Gonzalez from Facultad de Ciencias Exactas, for helping us during the realization of the clotting tests.

## References

Abugoch, L. E. (2006). *Relathionships between structure and functionality of amaranth glutelins and protein isolates (Amaranthus hypochondriacus)* (PhD thesis). Universidad Nacional de La Plata.

Adler-Nissen, J. (1979). Determination of degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of Agricultural and Food Chemistry, 12*, 605–623.

American Dietetic Association. (1999). Position of the American Dietetic Association. Functional foods. *Journal of American Dietetic Association, 99*, 1278–1285.

Barrio, D. A., & Añón, M. C. (2010). Potential antitumor properties of a protein isolate obtained from the seeds of *Amaranthus mantegazzianus*. *European Journal of Nutrition, 49*, 73–82.

Bressani, R. (1994). Composition and nutritional properties of amaranth. In O. Paredes-López (Ed.), *Amaranth. Biology, chemistry and technology* (pp. 185–206). Boca Ratón: CRC Press, Inc.

Castellani, O. F., Martínez, N. E., & Añón, M. C. (2000). Globulin-P structure modifications induced by enzymatic proteolysys. *Journal of Agricultural and Food Chemistry, 48*, 5624–5629.

Condés, M. C., Scilingo, A., & Añón, M. C. (2009). Characterization of amaranth proteins modified by trypsin proteolysis. Structural and functional changes. *Food Science and Technology, 42*, 963–970.

Han, J., & Lim, S. (2004). Structural changes in corn starches during alkaline dissolution by vortexing. *Carbohydrate Polymers, 55*, 193–199.

Jung, W. K., Je, J. Y., Kim, H. J., & Kim, S. K. (2001). A Novel anticoagulant protein from *Scapharca broughtonii*. *Journal of Biochemistry and Molecular Biology, 35*, 199–205.

Jung, W. K., Jo, H. Y., Qian, Z. J., Jeong, Y. J., Park, S. G., Choi, I. W., et al. (2007). A novel anticoagulant protein with high affinity to blood coagulation factor Va from *Tegillarca granosa*. *Journal of Biochemistry and Molecular Biology, 40*, 832–838.

Jung, W. K., & Kim, S. K. (2009). Isolation and characterisation of an anticoagulant oligopeptide from blue mussel, *Mytilus edulis*. *Food Chemistry, 117*, 687–692.

Laudano, A. P., & Doolittle, R. F. (1978). Synthetic peptide derivatives that bind to fibrinogen and prevent the polymerization of fibrin monomers. *Proceedings of National Academy of Science, 75*, 3085–3089.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*, 265–275.

Martínez, N. E., & Añón, M. C. (1996). Composition and structural characterization of amaranth proteins isolates. An electrophoretic and calorimetric study. *Journal of Agricultural and Food Chemistry, 44*, 2523–2530.

Martínez, N. E., Castellani, O. F., & Añón, M. C. (1997). Common molecular features among amaranth storage proteins. *Journal of Agricultural and Food Chemistry, 45*, 3832–3839.

Mendonça, S., Saldiva, P. H., Cruz, R. J., & Arêas, J. A. G. (2009). Amaranth protein presents cholesterol-lowering effect. *Food Chemistry, 116*, 738–742.

Nkonge, Ch, & Ballance, M. (1982). A sensitive colorimetric procedure for nitrogen determination in micro-Kjeldahl digests. *Journal of Agricultural and Food Chemistry, 30*, 416–420.

Orsini Delgado, M. C., Tironi, V. A., & Añón, M. C. (2011). Antioxidant activity of amaranth protein or their hydrolysates under simulated gastrointestinal digestion. *Food Science & Technology, 44*, 1752–1760.

Paredes-López, O. (1994). *Amaranth: Biology, chemistry and technology*. CRC Press.

Quiroga, A. V., Martínez, E. N., Rogniaux, H., Geairon, A., & Añón, M. C. (2010). Amaranth (*Amaranthus hypochondriacus*) vicilin subunit structure. *Journal of Agricultural and Food Chemistry, 58*, 12957–12963.

Segura-Nieto, M., Barba de la Rosa, A., & Paredes-López, O. (1994). Biochemistry of amaranth proteins. In O. Paredes-López (Ed.), *Amaranth. Biology, chemistry and technology, chapter 5*. Boca Ratón: CRC Press.

Schägger, H. (2006). Tricine-SDS-PAGE. *Nature Protocols, 1*, 16–22.

Tang, C.-H., & Wang, X.-Y. (2010). Physicochemical and structural characterization of globulin and albumin from common buckwheat (*Fagopyrum esculenum* Moench) seeds. *Food Chemistry, 121*, 119–126.

Vecchi, B., & Añón, M. C. (2009). ACE inhibitory tetrapeptides from *Amaranthus hypochondriacus* 11S globulin. *Phytochemistry, 70*, 864–870.

Yang, W. G., Wang, Z., & Xu, S. Y. (2007). A new method for determination of antithrombotic activity of egg white protein hydrolysate by microplate reader. *Chinese Chemical Letter, 18*, 449–451.

Yemm, E. W., & Willis, A. D. (1954). The estimation of carbohydrates in plant extracts by anthrone. *Biochemical Journal, 57*, 508–514.

Zhang, S. B., Wang, Z., & Xu, S. Y. (2008). Antioxidant and antithrombotic activities of rapeseed peptides. *Journal of American Oil Chemistry Society, 85*, 521–527.