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Potential antithrombotic activity detected in amaranth proteins and its hydrolysates

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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.

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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).

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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:

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

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