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Amaranth lectin presents potential antitumor properties

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ABSTRACT

The possibility of reducing the risk of cancer is one of the most important challenges facing scientists. In this work we demonstrate that the amaranth lectin presents potential antitumor activity and we propose a mechanism of action. Our results indicate that the albumins and a hydrophobic fraction (MPI-h) from the *Amaranthus mantegazzianus* protein isolate (MPI) are capable of inhibiting UMR106 rat osteosarcoma-derived cell proliferation. Further fractionation of the hydrophobic fraction and LC-MS/MS analyses of the tryptic peptides showed the presence of lectin fragments in one active fraction. When a partially purified *A. mantegazzianus* lectin and the commercial *Amaranthus caudatus* lectin were tested as cell-proliferation inhibitors, both lectins showed high activity with an $IC_{50} = 0.1$ mg/mL and 0.08 mg/mL respectively; similar to MPI-h activity. Lectins would exert a cytotoxic effect followed by cell apoptosis, in addition to inhibiting cell adhesion.

1. Introduction

Food and nutrition are important factors that exert a strong influence on the development of several chronic diseases, including cancer. Moreover, there is increased evidence that specific dietary patterns, foods and drinks, and dietary constituents (proteins, peptides and secondary metabolites) can protect against cancer (World Cancer Research Fund, 2007). Among the bioactive food components, some proteins and peptides from different sources are under clinical or preclinical studies to be used as alternative therapies to conventional cancer treatments (Bhutia & Maiti, 2008; Perez Espitia et al., 2012; Udenigwe & Aluko, 2012). Peptides and proteins exert their action through different mechanisms covering all stages of cancer development including initiation, promotion and progression (Bhutia & Maiti, 2008; González de Mejia & Dia, 2010). There are some well known examples of peptides and proteins derived from plants with a recognized antitumor activity among them we can mention lunasin and Bowman Birk inhibitor from soybean (Hernández-Ledesma, Hsieh, & de Lumen, 2009; Kobayashi, 2013), and some plant lectins (González de Mejía & Prisecaru, 2005). Amaranth, an ancient crop with a protein content (12–17 g/100 g flour) higher than that of most cereals, has been reported as an important source of bioactive components (Caselato-Sousa & Amaya-Farfán, 2012; Rastogi & Shukla, 2013). More recently, bioactive peptides derived from amaranth proteins have been described. They have shown different biological activities such as antimicrobial, antioxidant, and antihypertensive (Caselato-Sousa & Amaya-Farfán, 2012; Fritz, Vecchi, Rinaldi, & Añón, 2011; Lipkin et al., 2005; Orsini Delgado, Tironi, & Añón, 2011; Quiroga, Aphalo, Ventureira, Martínez, & Añón, 2012). It has been also reported a potential hypocholesterolemic effect associated to amaranth proteins (Mendonça, Saldiva, Cruz, & Arêas, 2009).

Silva-Sánchez et al. (2008) have found a lunasin-like peptide in the glutelin fraction from *Amaranthus hypochondriacus* with potential antitumor properties. In a previous work Barrio and Añón (2010) reported an antiproliferative activity in *Amaranthus mantegazzianus* protein isolate and presented its putative mechanism of action in different tumor and non tumor cell lines. Based on these findings, the objective of this work was to select and identify the proteins/peptides from the isolate that developed the potential antitumor activity. On this regard we isolated different amaranth protein/peptides fractions, determined their antiproliferative activity and the possible mechanism of action.

Abbreviations: MPI, *Amaranthus mantegazzianus* protein isolate; MPI-h, hydrophobic fraction; AB, disulphide-linked A–B; A, acidic polypeptide; B, basic polypeptide; M, propolypeptide.

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2. Materials and methods

2.1. Sample preparation

2.1.1. Plant materials

The seeds of *A. mantegazzianus* commercial variety (Pass cv Don Juan Inta Anguil, La Pampa) were harvested at Universidad Nacional de La Pampa, (Santa Rosa, Argentina). Flour was obtained as described previously Martínez and Añón (1996).

2.1.2. Preparation of protein isolates

The protein isolate (MPI) was prepared as described by Martínez and Añón (1996). Briefly, proteins were extracted at pH 9 from the defatted flour and precipitated at pH 5. The precipitate was suspended in water, neutralized and freeze-dried.

2.1.3. Protein fractionation

Amaranth protein fractions were prepared according to the procedure reported elsewhere (Martínez & Añón, 1996). The defatted amaranth flour was successively treated three times with: water; buffer A (0.0325 mol/L K_2HPO_4, 0.0026 mol/L KH_2PO_4, pH 7.5, 0.4 mol/L NaCl); water and 0.1 mol/L NaOH to extract albumins, globulins, globulin-P and glutelins respectively. Each extraction step, at a ratio of 10 mL solvent/g flour, was performed at room temperature, and after each treatment, the extraction residue was separated by centrifugation at 9000*g* for 20 min at room temperature. Globulin-P, globulin and glutelins were isolated from the corresponding supernatants by precipitation at pH 6. The precipitates were suspended in water, neutralized and freeze-dried.

2.1.4. Preparation of a hydrophobic fraction

The isolate was treated with dimethyl sulfoxide:H_2O (50:50, v/v) under continuous stirring for 1 h. Treatment was performed at room temperature in a 3 g isolate/100 mL solvent ratio, and then centrifuged at 22,000*g* for 30 min at room temperature. The protein fraction of the supernatant was precipitated with 6 mol/L HCl, afterward it was centrifuged at 12,000*g* for 10 min at 4 °C, and the pellet was suspended in water, neutralized and freeze-dried. This was called MPI-h.

2.1.5. Amaranth lectins

Amaranthus caudatus lectin was purchased from Vector laboratories (Burlingame, CA, USA).

Partially purified *Amaranth* lectin was prepared as described by Rinderle, Goldstein, Mattag, and Ratcliffell, (1989) with minor modifications. Briefly, the defatted flour was treated three times with phosphate-buffered saline (PBS: 0.011 mol/L KH_2PO_4, 0.026 mol/L Na_2HPO_4, 0.115 mol/L NaCl, pH 7.4) at room temperature for 1 h. Proteins were precipitated with 1.2–3.2 mol/L $(NH_4)_2SO_4$ (4 °C). The precipitated proteins were dissolved in 0.020 mol/L tris(hydroxymethyl)aminomethane (Tris), pH 8.1, and dialyzed extensively against the same buffer. After centrifugation the protein sample was applied to a MonoQ 5/50 GL (GE-Healthcare, Uppsala, Sweden) linked to an FPLC System (Pharmacia LKB, Uppsala, Sweden) equilibrated and washed with 0.020 mol/L Tris, pH 8.1. Protein peaks in the washing fraction were pooled and analyzed by SDS-PAGE. Results showed a single band of approximately 35 kDa.

2.1.6. Preparation of protein solutions

Dispersions of the different protein fractions in Dulbecco's modified Eagle's medium (DMEM, GibcoBRL, Buenos Aires, Argentina) (10–100 mg/mL) were gently stirred for 1 h at room temperature and centrifuged at 22,000*g* for 30 min at room temperature. The supernatant was then sterilized by filtration through a 0.22 polypropylene membrane (Millipore, Billerica, MA, USA) and the soluble protein was determined by the Kjeldahl method ($n \times 5.85$). Dilutions of these samples were used to the biological assays.

2.2. Structural characterization

2.2.1. SDS-tris/HCl and SDS-tris/Tricine–polyacrylamide gel electrophoresis (SDS-PAGE and Tricine–SDS-PAGE)

Runs were performed in minislabs (Bio-Rad, Hercules, CA, USA). In SDS-PAGE system (Laemmli, 1970) gels of 12 g acrylamide/100 mL of resolving gel; 4 g acrylamide/100 mL of stacking gel were used. In SDS-PAGE–Tricine system (Schägger, 2006) gels of 4 g acrylamide/100 mL of stacking gel, 10 g acrylamide/100 mL of spacer gel, and 16 g acrylamide + 0.6 mol urea/100 mL of separating gel were used. Low molecular weight calibration kit (MW 14.1–97 kDa) (Pharmacia LKB) and polypeptide SDS-PAGE standards (MW 1.4–26.6 kDa) (Bio-Rad) were used as molecular markers for SDS-PAGE and Tricine–SDS-PAGE. Gels were fixed and stained with Coomassie brilliant Blue.

2.2.2. Two-dimensional electrophoresis (IEF–SDS-PAGE)

Samples were analyzed by two-dimensional electrophoresis as described by Quiroga, Martínez, Rogniaux, Geairon, and Añón (2010). Isoelectric focusing (IEF) was run using 7-cm linear immobiline pH gradient (IPG) strips (pH 3–10) in the IPGphor system (GE-Healthcare). Samples were dissolved in the rehydration buffer and the IPG strips were rehydrated with these samples. Following IEF, the gel strips were incubated with equilibration buffer and were placed onto 15 g acrylamide/100 mL of resolving gels and were run in minislabs (Bio-Rad). All gels were fixed and stained with Coomassie Brilliant Blue.

2.2.3. Fast protein liquid chromatography (FPLC). Gel filtration

The MPI-h was analyzed at room temperature in a Superdex 75 HR column using a FPLC System (Pharmacia, LKB). Samples (4 mg of protein) were dissolved in 0.2 mL of buffer (0.0351 mol/L K_2HPO_4, pH 8, μ 0.1) and were eluted with the same buffer at a flow rate of 0.2 mL/min. Fractions of 0.5 mL were collected, and the elution profile (absorbance at 280 nm) was obtained. The column was calibrated with gel filtration calibration kits (GE-Healthcare). Curves were processed and data were evaluated using the Pharmacia AB, FPLC director and FPLC assistant software.

2.2.4. High performance liquid chromatography (HPLC). Reverse-phase chromatography (RP-HPLC)

The runs were carried out in a Waters 600 E HPLC Multisolvent Delivery System equipped with a Water 717 Plus Autosampler and a Waters 996 Photodiode Array Detector (Waters Corporation, Milford, MA, USA).

SunFire prep C8, 5 μm ST 10/250 (Waters Corporation) column was equilibrated with solvent A (water:acetonitrile, 98:2 (v/v); 0.065 mL/100 mL Trifluoracetic Acid, TFA in water). Two milliliters of 3 mg/mL MPI-h in 0.0351 mol/L K_2HPO_4, pH 8, μ 0.1 was injected. Samples were eluted with solvents A and B (water:acetonitrile, 35:65 (v/v); 0.065 mL/100 mL TFA in water) in a linear gradient of B from 0 to 100% in 55 min at a flow rate of 5.2 mL/min. Detection was performed at 210 and 280 nm. Fractions (0.6 min) were collected manually from 5 runs, pooled and freeze-dried.

2.2.5. Mass spectrometry (MS)

MS 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).

The protein spots were prepared according to the procedure reported in Quiroga et al. (2012). Protein identification was first attempted by the peptide mass-fingerprint approach by means of MALDI-TOF mass spectrometry. Analyses were performed with a MALDI LR instrument equipped with a conventional 337-nm laser (Micromass/Waters, Manchester, UK). Mass-data acquisitions were piloted by the Mass Lynx software (Micromass/Waters). This gave a fingerprint of the samples and allowed to rapidly checking which ones were similar.

LC-MS/MS analyses were further performed, through the use of a Switchos-Ultimate II capillary LC system (LC Packings/Dionex, Amsterdam, The Netherlands) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF Global, Micromass/Waters). Chromatographic separation was conducted on a reverse-phase capillary column (Pepmap C18, 75-μm × 15-cm, LC Packings) with a linear gradient from 2 to 40 mL/100 mL acetonitrile in 50 min, followed by an increase to 50 mL/100 mL acetonitrile within 10 min, at a flow rate of 0.2 μL/min.

Mass-data acquisitions were piloted by the Mass Lynx software (Micromass/Waters): the MS data were recorded for 1 s on the mass-to-charge (m/z) range 400–1500, after which time the three most intense ions (doubly, triply, or quadruply charged) were selected and fragmented in the collision cell (MS/MS measurements).

2.2.5.1. Protein identification—databank searching from LC-MS/MS data. Raw data were processed by means of the Protein Lynx Global Server v. 2.1 software (Micromass/Waters). Protein identification was performed by comparing the collected LC-MS/MS data against Uniprot databank restricted to Viridiplantae species (release April, 2010; 751,663 sequences). The mass tolerance was set at 150 ppm for parent ions (MS mode) and 0.3 Da for fragment ions (MS/MS mode), and one missed cut per peptide was allowed. Databank searches were performed through the use of the Mascot server v. 2.2 program (Matrix Science, London, UK).

2.2.5.2. Validation of protein identifications. For MS/MS experiments, the validation of proteins was first performed by setting the requirements to a minimum of two MS/MS spectra matching the databank sequence with individual MASCOT ion scores above the significance threshold (threshold score of 43, $p < 0.05$).

2.3. Biological characterization

2.3.1. Cell culture

According to Barrio and Añón (2010) results UMR106 rat osteosarcoma-derived cells were the most sensitive in the antiproliferative activity determinations, therefore UMR106 cell line (ATCC, Manassas, VA, USA) was chosen for this work analyses. Cells were grown in DMEM supplemented with penicillin (100 U/mL) (Roemmers, Buenos Aires, Argentina), streptomycin (100 μg/mL) (Roemmers), and FBS (0.1 mL/mL) (GibcoBRL) in a humidified atmosphere of air plus 5% CO_2 at 37 °C. The cells (70–80% confluence) were harvested using trypsin (0.1 g/100 mL), 1 mmol/L EDTA in Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS) diluted 1:10 (v/v) and sub-cultured as mentioned above.

2.3.2. Cell-proliferation assay

A mitogenic bioassay was carried out as described by Okajima et al. (1992) with some modifications. Stated in brief, cells were grown in 48-well plates. When the cultures reached 60% confluence, the monolayers were washed twice with serum-free DMEM and incubated with DMEM with FBS (0.01 mL/mL) (basal condition) or different dilutions of the test samples dissolved in DMEM with FBS (0.01 mL/mL). After 24-h incubation, the monolayers were washed with PBS and fixed with 0.05 mL/mL glutaraldehyde in PBS at room temperature for 10 min, then stained with 5 mg/mL of crystal violet in water:methanol 75:25 (v/v) for 10 min. The dye solution was then discarded and the plate washed with water and dried. The dye taken up by the cells was extracted with 0.5 mL/well of 0.1 mol/L glycine/HCl buffer, pH 3.0 in 30 mL/100 mL aqueous methanol and transferred to test tubes. The absorbance at 540 nm was read at a convenient sample dilution. Under these conditions, the colorimetric bioassay strongly correlated with cell proliferation as measured by cell counting in a Neubauer chamber.

2.3.3. Cell morphology

Cells grown on glass coverslips were incubated in DMEM with FBS (0.01 mL/mL) (basal condition) or with different dilutions of the MPI-h plus FBS (0.01 mL/mL final concentration) for 24 h. The cultures were then fixed and stained with Giemsa. The stained samples were finally examined with light microscopy and photographs taken for later evaluation.

2.3.4. Cytoskeletal rearrangement

To examine actin filaments, they were stained with fluorescence isothiocyanide- (FITC–phalloidin). Cells were grown on glass coverslips to 70% confluence and were then incubated for 24 h at 37 °C with DMEM plus FBS (0.01 mL/mL) (basal condition) or different dilutions of the MPI-h plus FBS (0.01 mL/mL final concentration). Next the cells were fixed, permeabilized with absolute ethanol (chilled to −20 °C) for 4 min at room temperature and washed with PBS. After an initial washing, the cells were incubated with FITC–phalloidin for 2 h, washed again, and mounted on slides. The cells labeled with green fluorescence were examined by means of a fluorescence microscope and photographs taken for later evaluation.

2.3.5. Cytotoxicity assays

2.3.5.1. Neutral red assay. The neutral red accumulation assay was performed according to Borenfreund, Babich, and Martin-Alguacil (1988). A total of 5×10^4 cells/well was plated in 48-well tissue–culture plates. Cells were treated with different concentrations of the MPI-h for 24 h at 37 °C in an atmosphere of air plus 5% CO_2. After treatment, the medium was replaced by one containing 100 μg/mL NR dye and the cells were incubated for 3 h. The dye solution was then discarded and the cells were washed with PBS (pH 7.4). The dye taken up by the lysosomes of viable cells was extracted with 100 μL of ethanol:water:acetic acid, 50:49:1 (v/v). The plates were shaken for 10 min, and the absorbance of the solution in each well was measured at 540 nm. Optical density was plotted as percent of control (assuming data obtained in the absence of the MPI-h as 100%).

2.3.5.2. MTT assay. The assay was performed as previously described by Mosmann (1983). Briefly, 5×10^4 cells/well were seeded in a 48-multiwell dish, allowed to attach for 24 h and treated with different concentrations of the MPI-h for 24 h. After this treatment, the medium was changed and cells were incubated with 0.5 mg/mL MTT under standard culture conditions without phenol red for 3 h. Cell viability was marked by the conversion of the tetrazolium salt (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide, MTT) to a colored formazan by mitochondrial dehydrogenases. Color development was measured in a Microplate Reader Synergy HT (BioTeK Instruments, Winooski, VT, USA) at 570 nm after cell lysis in dimethyl sulfoxide (100 μL/well). Optical density was plotted as percent of control (assuming data obtained in the absence of the MPI-h as 100%).

2.3.6. Cell adhesion

The cell-adhesion assay was performed according to McCarty, Uemura, Etcheverry, and Cortizo (2004) with some modifications. Cells were plated in DMEM with FBS (0.1 mL/mL) in 24-well plates at a seeding density of 10^5 cells/well in the presence of different concentrations of the MPI-h The cells were incubated for 4 h at 37 °C to adhere to the surface. Each well was then washed with PBS and the crystal-violet assay was performed to determine the final cell number.

2.4. Statistical methods

At least five replicates of the MPI-h were obtained and three independent determinations were performed for each assay. The PROBIT method was used to calculate the concentration that inhibits 50% of the cell proliferation (IC_{50}). The results were expressed as the mean ± SD. Multifactor analysis of variance (ANOVA) of the variables was performed by means of Statgraphics Plus, a software package from Statgraphics Corp. (Rockville, MD, USA). Fisher's protected LSD ($p < 0.02$) values were calculated for the appropriate data.

3. Results

As previously reported (Barrio & Añón, 2010), an amaranth protein isolate was capable to decrease the proliferation of rat tumor cells of line UMR106. To find the protein component that developed the antiproliferative activity, we purified the protein fractions that comprised the isolate; albumins, globulins and glutelins. We also studied a MPI-h obtained from the protein isolate.

3.1. Structural characterization

3.1.1. SDS-PAGE and Tricine–SDS-PAGE

Samples dissolved in culture medium were analyzed. SDS-PAGE patterns are shown in Fig. 1a and b. The MPI (Fig. 1a) was composed by 11S-globulin and globulin-P (AB: disulphide-linked A–B, A: acidic polypeptide, B: basic polypeptide, M: propolypeptide and MM > 97 kDa: polypeptides with molecular masses higher than 97 kDa, Martínez & Añón, 1996), 7S-globulin (polypeptides indicated by black arrows (Martínez & Añón, 1996; Quiroga et al., 2010), and albumins (polypeptides that are found throughout the profile, Segura-Nieto, Barba de la Rosa, & Paredes-López, 1994). After treatment with 2-mercaptoethanol (Fig. 1a) the polypeptides A and B were present. They came from the dissociation of the AB and the high molecular mass polypeptides. The electrophoretic pattern of the MPI-h (Fig. 1b) showed a main band of 35.5 kDa (black arrow) and bands of approximately 77 and 60 kDa (grey circle). Bands of 29 and 25 kDa (grey circle) were also present. The SDS-PAGE pattern of the MPI-h treated with 2-mercaptoethanol showed an increment of the polypeptides of 77, 60 and 35.5 kDa suggesting the presence of species joined by disulfide bonds.

The Tricine–SDS-PAGE pattern is shown in Fig. 1c. The isolate contained polypeptides higher than 45 kDa (grey circle), polypeptides of 35, 31 and 21 kDa (black arrows) and polypeptides lower than 16.9 kDa (grey circle) (Fig. 1c, MPI). The MPI-h also showed the polypeptides higher than 45 kDa and those of 31 and 21 kDa (large black arrows), but, in a lower proportion, it showed polypeptides of 35 and approximately 26 kDa (small black arrows) (Fig. 1c, MPI-h). In the presence of 2-ME the proportion of polypeptides higher than 45 kDa decreased, but the other bands showed no difference.

3.1.2. Gel filtration

The FPLC profile of the MPI-h (Fig. 2a) showed species higher than 70 kDa (V0), other of approximately 36 kDa (I) and species smaller than 6 kDa (II and III peaks).

The SDS-PAGE patterns of species V0 and I showed a main band of approximately 35 kDa (Fig. 2b line V0 + I, black arrow) and high molecular weight polypeptides (Fig. 2b line V0 + I, grey circle). By treatment with 2-mercaptoethanol the intensity of high molecular weight polypeptides decreased (Fig. 2b line (V0 + I) + 2-ME).

The SDS patterns of peaks II and III showed no bands probably due to the low amount of protein in the samples (result not shown).

3.2. Biological activity

3.2.1. Inhibition of cell proliferation

The effect of the protein fractions, albumins, globulins, and glutelins, and the MPI-h on cell proliferation was determined and compared with the effect of the MPI (Table 1). The effect of

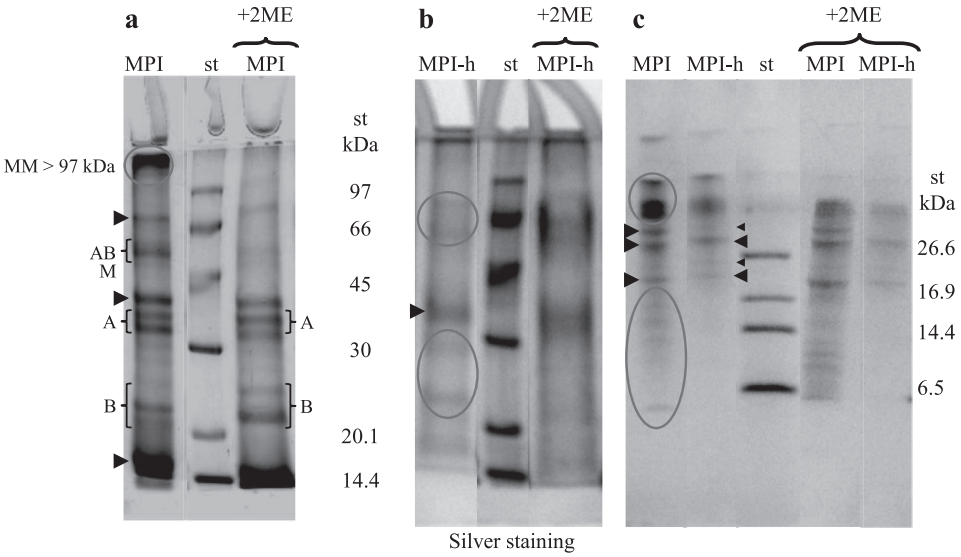

Fig. 1. Electrophoretic patterns of isolate (MPI) and MPI-h: a) SDS-PAGE: MPI and MPI + 2-ME (reducing conditions). Polypeptides from 11S-globulin and globulin-P are indicated as: AB: disulphide-linked A–B, A: acidic polypeptide, B: basic polypeptide, M: propolypeptide and MM > 97 kDa: polypeptides with molecular masses higher than 97 kDa. Polypeptides from 7S-globulin are indicated by black arrows, b) SDS-PAGE: MPI-h, and MPI-h + 2-ME (reducing conditions). St: Standard proteins; st-kDa: molecular masses of the standard proteins, c) Tricine–SDS-PAGE: MPI and MPI-h; MPI and MPI-h + 2-ME (reducing conditions). St: standard proteins; st-kDa: molecular masses of the standard proteins.

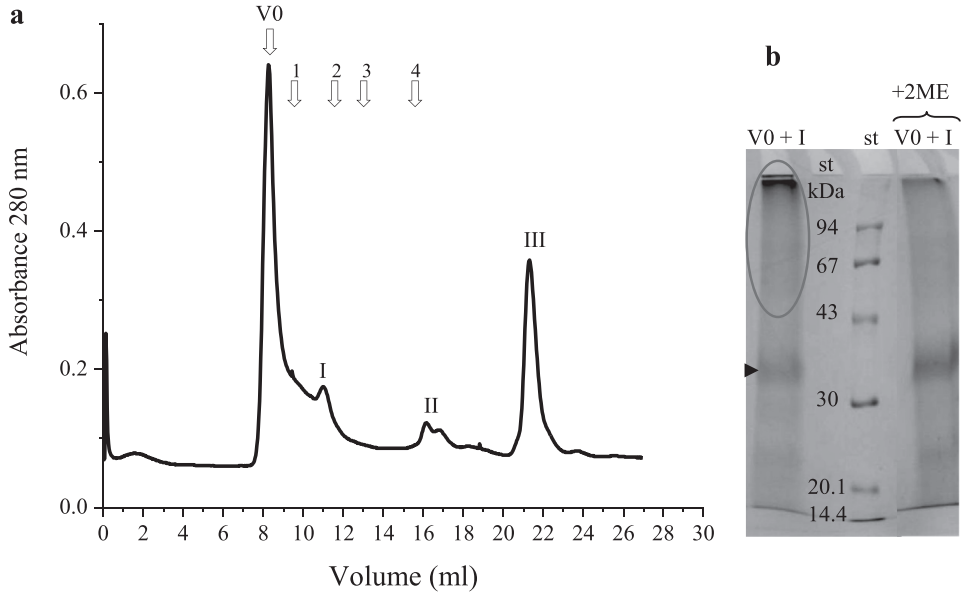

Fig. 2. a) Gel filtration profile of the MPI-h. White arrows indicate the elution volumes of standard proteins: V0: Void volume, 1: Albumin (66 kDa); 2: Carbonic anhydrase (29 kDa); 3: Cytochrome *c* (12.4 kDa); 4: Apotimin (6.5 kDa), b) SDS-PAGE: (V0 + I), (V0 + I) + 2-ME (reducing conditions). St: standard proteins; st-kDa: standard molecular masses.

globulin-P on cell proliferation could not be determined due to its low solubility in the culture medium. Globulins and glutelins presented a low effect while albumins exhibited the highest activity among the protein fractions. MPI showed an effect higher than globulins and glutelins but lower than albumins. The most potent inhibitor was the MPI-h with an IC_{50}: 0.1 mg/mL, 10 times lower than the MPI. Considering these results we chose MPI-h to continue working with.

3.2.2. Changes in cell morphology

Fig. 3a shows the morphological characteristics of the UMR106 osteosarcoma line. These cells exhibited a polygonal morphology with well stained irregular-shaped nuclei and a well defined vacuole-containing cytoplasm. Cells were interconnected by lamellar processes. Fig. 3b shows that after 24 h of incubation with 0.1 mg/mL MPI-h, condensation in the nucleus and cytoplasm took place. Under this condition the number of processes between the cells had diminished along with the number of cells per field. Besides, when the cells were incubated with 0.2 mg/mL MPI-h, cellular pyknosis and fragmented nuclei typical of apoptotic cells were shown, and the cell borders were also poorly defined. The cells exhibited a dense nucleus surrounded by a very small and highly condensed cytoplasm (data not shown).

Table 1
Inhibition of UMR106 cell line proliferation.

Protein fractions	IC_{50} (mg/mL)	IC_{50} (μMol/L)
MPI	1.000 ± 0.050[a]	–
Albumins	0.500 ± 0.050[b]	–
Globulins	>2.000 ± 0.100[c]	–
Glutelins	>2.000 ± 0.100[c]	–
Globulin-P	ND	–
MPI-h	0.100 ± 0.010[e]	–
MPI-h (I)	0.060 ± 0.010[f]	–
MPI-h (II)	0.100 ± 0.030[e]	–
MPI-h (III)	0.050 ± 0.001[fg]	–
MPI-h (IV)	0.040 ± 0.001[g]	–
MPI-h (V)	0.020 ± 0.001[h]	–
c-lectin	0.080 ± 0.020[e]	1.200 ± 0.300
pp-lectin	0.100 ± 0.010[e]	1.510 ± 0.150

IC_{50}, protein concentration that inhibits the 50% of cell proliferation; IC_{50} (μMol/L), protein concentration that inhibits the 50% of cell proliferation considering a molecular weight of 66 kDa; ND, not determinate; MPI, protein isolate; MPI-h, hydrophobic fraction; MPI-h (I–V), RP-HPLC fractions; c-lectin, commercial lectin; pp-lectin, partially purified lectin.
Different letters in the IC_{50} (mg/mL) column means significant differences ($p < 0.05$). $n = 4$ (number of replications).

3.2.3. Changes in cytoskeletal proteins

Fig. 3c shows the characteristics of the actin filaments in the non treated cells. The cells display an organized architectural distribution of the micro-fibrils: the actin microfilaments are normally oriented in the direction of the main axis of the cells. When the UMR106 cells were exposed to different concentrations of the MPI-h for 24 h, the regularity of the micro-fibrils pattern was lost. At 0.1 mg/mL MPI-h a partial disorganization of the actin microfilaments in the cytoplasm was shown together with an alteration in the shape of the cells (Fig. 3d). This result is in accordance with the results of morphological alterations as well as with the results in the inhibition of cell-proliferation analyses.

3.2.4. Cytotoxicity assays

With the aim of evaluating the cytotoxic effects of the MPI-h we have carried out NR and MTT assays. The NR assay is based on the lysosomal uptake of the neutral red dye by viable cells. With the MTT assay viable cells are determined by measuring the mitochondrial succinic dehydrogenase activity. Results obtained by the two methods are shown in Fig. 4a. Percentage of dye taken up by viable cells with respect to the non treated control is plotted against the concentrations of the MPI-h in the incubations. This figure showed the cytotoxic effect caused after MPI-h treatment, detected by their mitochondrial succinic dehydrogenase activity as well as by their lysosomal neutral red uptake. These results indicated that the MPI-h affected the cells viability in a dose-dependent way.

3.2.5. Inhibition of cell adhesion

Cell adhesion is an essential aspect of cell survival, in particular for metastatic tumor cells (Morris, Morris, Kennedy, & Sweeney,

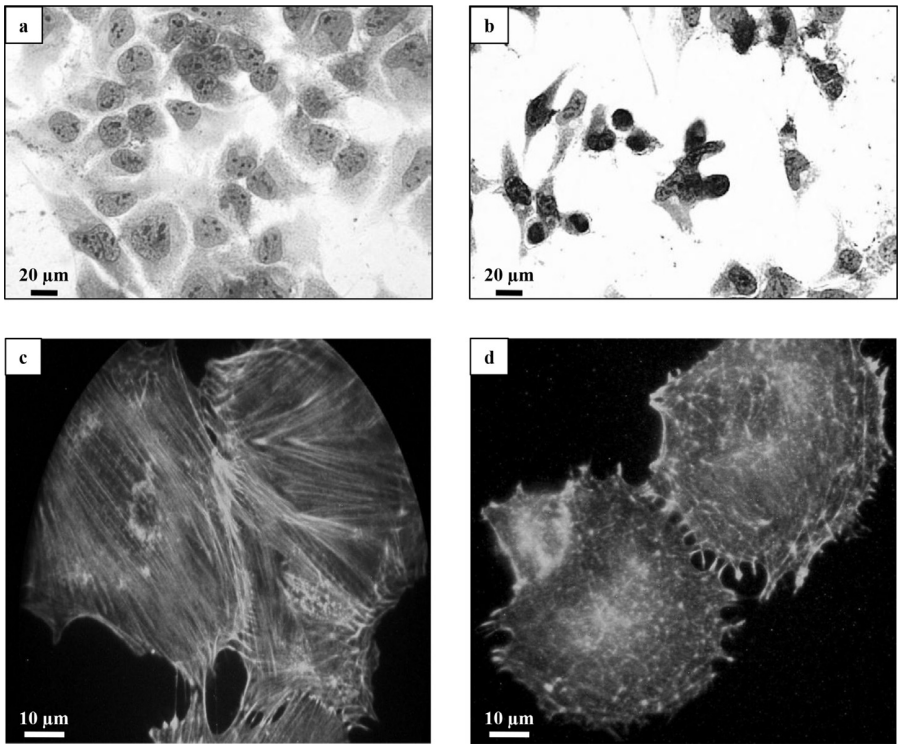

Fig. 3. Changes in cell morphology: a) UMR106 osteosarcoma cells incubated for 24 h, b) UMR106 osteosarcoma cells incubated with 1 mg/mL MPI-h for 24 h. Cells were fixed with methanol and stained with Giemsa. Light microscopy photographs, magnification 63×. Changes in cytoskeletal proteins: c) UMR106 osteosarcoma cells incubated for 24 h, d) UMR106 osteosarcoma cells incubated with 1 mg/mL MPI-h for 24 h. Cells were fixed with cold ethanol and stained with FITC–phalloidin. Fluorescent microscopy photographs, magnification 63× and an additional digital zoom of 3×.

2005). We evaluated the action of the MPI-h on cell adhesion in the UMR106 cell-culture model. Fig. 4b showed that the viable cells that adhere to the plate after MPI-h treatment decreased with the increase of the MPI-h concentration. This result indicated that the MPI-h inhibited cell adhesion in a dose-dependent way, exhibiting an IC_{50} value at 0.07 ± 0.001 mg/mL, as determined by the PROBIT method.

3.3. Characterization and identification of peptides/proteins

3.3.1. Reverse-phase chromatography. RP-HPLC

The MPI-h was run and fractionated by RP-HPLC (Fig. 5a) and the chromatographic fractions were analyzed by Tricine–SDS-PAGE (Fig. 5b).

Fig. 5b shows that fraction I contained polypeptides of approximately 30 (P30) and 20 kDa (P20) (black arrows) and a group of polypeptides lower than 20 kDa (grey circle). Fractions II–V presented the same two polypeptides of approximately 30 and 20 kDa and also polypeptides higher than 45 kDa (grey circle). Fraction V pattern showed the 20 and 30 kDa polypeptides in a proportion higher than in the other fractions.

3.3.2. Inhibition of cell proliferation by different fractions from RP-HPLC

The IC_{50} of the RP-HPLC fractions are shown in Table 1 (MPI-h I–V[1]). All fractions were able to inhibit cell proliferation better than the MPI and fraction I, III–V even better than the MPI-h. Fraction V was the most potent inhibitor with an IC_{50}: 0.02 mg/mL, 50 times better than the IC_{50} of the MPI, so that the protein composition of this fraction was analyzed.

3.3.3. MS-analysis of tryptic fragments

The MPI-h and the chromatographic fraction V were resolved by two-dimensional electrophoresis (IEF/Tricine–SDS-PAGE, data not shown) and some of the spots (P30 and P20) were analyzed by LC-MS/MS. The fingerprint of the samples showed that P20 from fraction V (P20 (V)) and from the MPI-h (P20 (MPI-h)) were similar, while P30 from fraction V (P30 (V)) and from the MPI-h (P30 (MPI-h)) were different. Then we assessed the protein identity of P30 (V), P30 (MPI-h) and P20 (MPI-h) by LC-MS/MS by comparing the masses of collected peptides with reported sequences in the Uniprot databank pertaining to green plants.

Results showed that amaranth 11S-globulin peptides were present in P30 (V) as well as in P30 (MPI-h) (IQIVNDQGQSVFDEELSR; TSENAMFQSLAGR; QAFEDGFEWVSFK; TSENAMFQSLAGR; EFQQGNECQIDR; LTALEPTNR; ADVYTPEAGR; FNRPETTLFR; VQEGLHVIKPPSR; IQAEAGLTEVWDSNEQEFR; LLAESFGVSEEIAQK; CAGVSVIR; GERNTGNIFR). Besides, the three samples contained peptides belonging to an amaranth lectin (agglutinin); P30 (V) and P30 (MPI-h) contained the lectin peptides ETNEAAALFR; SIFQFPK; RYVTFK; ILDPLAQFEVEPSK; TYDGLVHIK, and P20 (MPI-h) contained the lectin peptide LSTDDWILVDGNDPR.

These results suggest that an amaranth lectin is involved in the antiproliferative activity developed by the amaranth isolate.

3.3.3.1. Inhibition of cell proliferation by amaranth lectins. *In vitro* antiproliferative activity of a partially purified amaranth lectin (pp-lectin) and a commercial lectin (c-lectin) was tested. Both samples inhibited cell proliferation in a dose-dependent way.

[1] MPI-h I–V: RP-HPLC fractions.

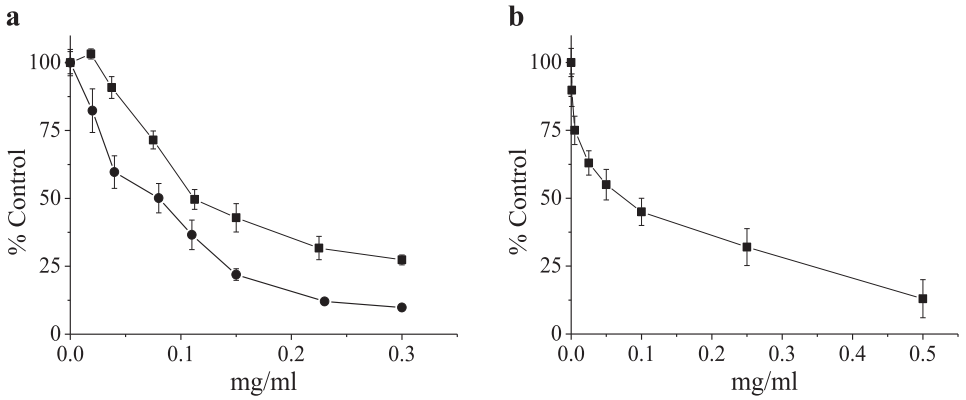

Fig. 4. a) Percentage with respect to control of cells with mitochondrial succinic dehydrogenase activity (- ● - MTT) or cells with neutral red uptake (- ■ - NR) treated by different concentrations of MPI-h. Results are expressed as % control of viable cells vs MPI-h concentration (mg/mL) and represent the mean ± SD, $n = 12$, b) Percentage with respect to control of cell that adhere to the plate in the presence of different concentrations of MPI-h. Results are expressed as % control of viable cells vs MPI-h concentration (mg/mL) and represent the mean ± SD of three independent experiments.

According to their IC_{50} (Table 1) they were 10 times more active than the protein isolate (MPI) and similar to the MPI-h. These results strongly suggest that the amaranth lectin was responsible for the antiproliferative activity found in the MPI-h.

4. Discussion

In this work we identified the peptides/proteins that were responsible of the previously reported MPI antitumor activity (Barrio & Añón, 2010). The MPI activity had been studied using different cell lines (cancerous and non-cancerous cell line from rat, mouse and human) (Barrio & Añón, 2010). This work results showed that the amaranth albumins and the MPI-h presented the capacity to inhibit the proliferation of UMR106 rat osteosarcoma-derived cells (Table 1). The antiproliferative capacity of the MPI-h was 10 times higher than that shown by the amaranth isolate and hydrolyzate (Barrio & Añón, 2010) whereas the RP-HPLC V fraction showed a capacity even higher, 50 times that of the isolate (Table 1).

Results of MS analyses of the tryptic fragments demonstrated the presence of amaranth 11S-globulin and lectin peptides in the MPI-h. Among other polypeptides, the MPI-h contained 31–35 kDa polypeptides which were able to aggregate forming near 70 kDa species (Fig. 2). Results showed that these polypeptides might belong to the amaranth lectin which was described as a homodimer of 66 kDa composed of 33–36 kDa single type subunits (Rinderle, Goldstein, Mattag, & Ratcliffell, 1989). The other polypeptides present in MPI-h might correspond to the globulins. However, globulins developed a lower activity than albumins and MPI-h (Table 1); therefore we proposed that the lectin polypeptides had the major responsibility of the cell-proliferation inhibition. When a partially purified and a commercial lectin was tested, they also showed antiproliferative activity thus supporting our hypothesis. However, we cannot exclude that any of the 11S-globulin peptides present in MPI-h also presented antiproliferative activity.

It is well documented that lectins have an antitumor effect (González de Mejía & Prisecaru, 2005; Kaur, Dhuna, Kamboj, Agrewala, & Singh, 2006; Lam & Ng, 2011; Liu, Bian, & Bao, 2010) though in some cases a pro-proliferative activity was detected (Ryder, Jacyna, Levi, Rizzi, & Rhodes, 1998; Yu, Milton, Fernig, & Rhodes, 2001). Most of the studies with different cell lines have assigned the antiproliferative activity to the induction of apoptosis

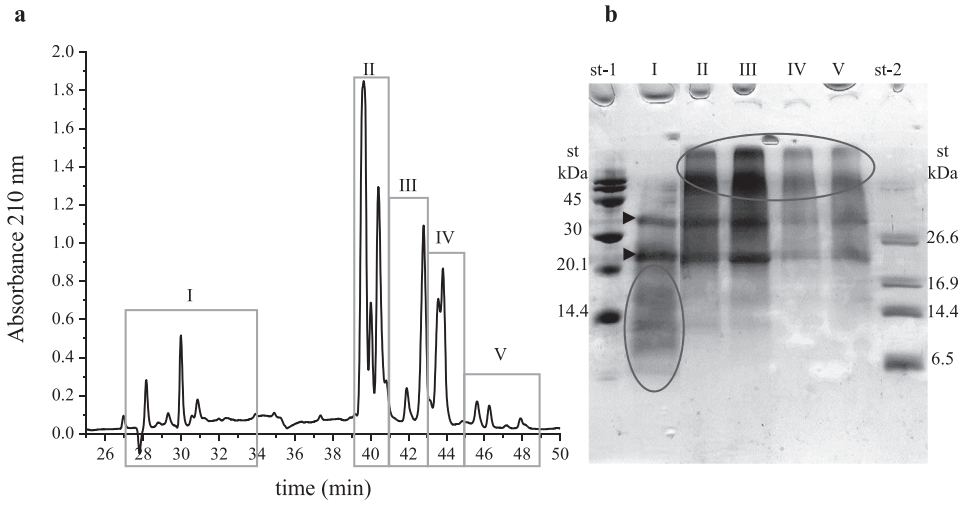

Fig. 5. a) RP-HPLC profile of the MPI-h, b) SDS-PAGE of peak fractions. St-1: Standard proteins; st-2: very low molecular weight standard proteins; st-kDa: molecular masses of the standard proteins.

mediated by different caspases (González de Mejia & Dia, 2010; Lam & Ng, 2011). These lectins would develop their effect by interacting with FAS receptors.

It has been reported that lectins resist gastrointestinal digestion, and can enter to the blood stream intact (González de Mejía & Prisecaru, 2005; Wang, Yu, Campbell, Milton, & Rhodes, 1998). On the other hand, in certain types of tumors it can be applied directly (local or subcutaneous administration) in order to avoid degradation during oral consumption (González de Mejía & Prisecaru, 2005; Pryme, Bardocz, Pusztai, & Ewen, 2006).

This work results suggested that the inhibition of the cellular proliferation by the MPI-h may be carried out by means of a cytotoxic effect shown by the mitochondrial and lysosomal alteration (Fig. 4a). This toxicity would promote apoptosis, which is expressed by cellular morphology modifications and by cytoplasmic actin filaments disorganization (Fig. 3). We have also demonstrated that MPI-h could inhibit cell adhesion 10 times better than MPI (Barrio & Añón, 2010). A decrease in the cellular adhesion process is very important in tumor propagation (metastasis) (Fig. 4b).

5. Conclusions

This work results strongly suggest that amaranth lectin was responsible for the antiproliferative activity of the MPI-h. The most active portion of the MPI-h (RP-HPLC fraction MPI-h (V), Table 1) was 5 times more potent than the purified lectin. This result can be explained considering that the lectin contained in MPI-h (V) was unfolded during the fractionation process. The unfolded lectin would present the active portion more exposed and therefore with more chances to interact with the cells. On the other hand we cannot exclude that any of the 11S-globulin peptides present in the more active fraction (MPI-h (V)) also presented antiproliferative activity.

At present, the activities of the MPI-h, the identified peptides and the purified lectin are being tested by *in vivo* assays.

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