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Bioactive Peptides in Amaranth (*Amaranthus hypochondriacus*) Seed

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Amaranth seeds are rich in protein with a high nutritional value, but little is known about their bioactive compounds that could benefit health. The objectives of this research were to investigate the presence, characterization, and the anticarcinogenic properties of the peptide lunasin in amaranth seeds. Furthermore, to predict and identify other peptides in amaranth seed with potential biological activities. ELISA showed an average concentration of 11.1 μg lunasin equivalent/g total extracted protein in four genotypes of mature amaranth seeds. Glutelin fraction had the highest lunasin concentration (3.0 μg/g). Lunasin was also identified in albumin, prolamin and globulin amaranth protein fractions and even in popped amaranth seeds. Western blot analysis revealed a band at 18.5 kDa, and MALDI-TOF analysis showed that this peptide matched more than 60% of the soybean lunasin peptide sequence. Glutelin extracts digested with trypsin, showed the induction of apoptosis against HeLa cells. Prediction of other bioactive peptides in amaranth globulins and glutelins were mainly antihypertensive. This is the first study that reports the presence of a lunasin-like peptide and other potentially bioactive peptides in amaranth protein fractions.

KEYWORDS: Amaranth; lunasin; anticancer peptides; antihypertensive peptides; bioactive peptides; HeLa cells

INTRODUCTION

Amaranth (*Amaranthus hypochondriacus*) is a traditional Mexican plant, which provides both grains and tasty leaves of high nutritional value, but it still remains as an underutilized crop. The National Academy of Sciences has stated that amaranth could be a grain with high potential for commercial exploitation because of its superior nutritional quality (*1*). The seed is high in protein (17%), and its amino acid composition is close to the optimum amino acid balance required in the human diet (*2*). The leaves also contain a high protein level (28 to 49%), unsaturated oil (45% linoleic acid), fiber (11 to 23%), and minerals such as potassium, iron, magnesium, and calcium (*2*). Aside from these nutritional components, amaranth seeds also contain other substances that play various biological roles in the diet, such as protease inhibitors, antimicrobial peptides, lectins, and antioxidant compounds (*3–5*). Aqueous extracts of *Amaranthus gangeticus* leaves have been reported to possess anticancer activity on liver, breast, and colon cancer cell lines (*6*). Vegetable parts of *Amaranthus tricolor* have been likewise found to have antitumor and anticell proliferation activities (*7*).

Lunasin is a unique 43 amino acid peptide whose cancer preventive properties have been demonstrated in a mammalian cell culture model and in a skin cancer mouse model against chemical carcinogens, oncogenes, and inactivators of tumor suppressor proteins (*8*). Its carboxyl-end contains nine Asp (D) residues, an **Arg-Gly-Asp** (RGD) cell adhesion motif, and a helix with structural homology to chromatin-binding proteins. Lunasin was found in the 2S albumin storage protein of soybean, and its appearance is reported to coincide with the initiation of mitotic arrest and DNA endoreduplication in the developing soybean cotyledon (*8–10*). The presence of lunasin in barley (*11*) and wheat (*12*) suggests the possibility that lunasin or lunasin-like compounds could be found in other grains. The objectives of this research were to investigate the presence, characterization, and the anticarcinogenic properties of the peptide lunasin in amaranth seeds and to predict and identify other peptides in amaranth seed with potential biological activities.

MATERIALS AND METHODS

Biological Material. Since lunasin concentrations vary depending on genotype and degree of maturity (*13*), mature amaranth (*Amaranthus*

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Table 1. ELISA Quantification of Lunasin-Like Protein in Mature Seeds of Amaranth in μg Lunasin Equivalent/g Extracted Protein[a]

	Criolla	DGETA	Gabriela	Nutrisol
	in total protein extract:[b]			
	12.0[c,a]	12.1[c,a]	9.5[c,a]	10.9[c,a]
	in protein fractions:[c]			
albumins	1.86[a,a]	1.85[a,a]	1.67[a,a]	1.67[a,a]
globulins 7S	1.85[a,a]	1.89[a,a]	1.52[a,a]	1.74[a,a]
globulins 11S	1.98[a,a]	1.80[a,a]	1.66[a,a]	1.77[a,a]
prolamins	1.54[a,a]	1.75[a,a]	1.39[a,a]	1.54[a,a]
glutelins	2.98[b,a]	3.01[b,a]	2.71[b,a]	2.82[b,a]

[a] Means not sharing a common superscript letter by column/row are significantly different at $p \leq 0.05$ by Tukey's multiple range tests. [b] Extracted at pH 7.4 as indicated in Materials and Methods. [c] Extracted under the conditions indicated in Materials and Methods for each protein fraction.

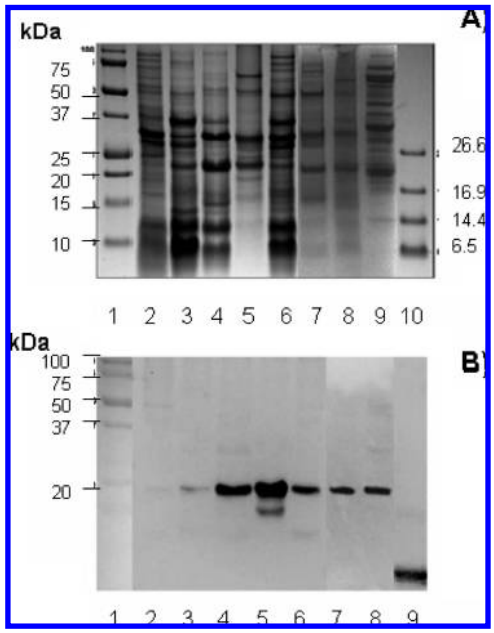

Figure 1. (**A**) Coomassie blue staining of amaranth total protein extract and protein fractions (10 μg of protein per well were loaded). (**B**) Western blot of amaranth proteins. Lanes: 1 and 10 = molecular weight markers, 2 = albumins; 3 = 7S globulins; 4 = 11S globulins; 5 = glutelins; 6 = total protein extract of mature amaranth seed; 7 = total protein extract of nonmature amaranth seeds; 8 = total protein extraction of popped amaranth seed; 9 = lunasin soybean enriched flour.

hypochondriacus) seeds from two commercial varieties (Criolla and Nutrisol) and two local noncommercial varieties (DGETA and Gabriela) were cultivated in Villa de Pozos, San Luis Potosi, Mexico, from September to December, 2002. The average size of mature seeds was 1.3 × 1.1 mm. Seeds were easily separated from the heads upon rubbing between the hands. Screening was performed in two commercial varieties and two new varieties of amaranth in an effort to introduce new crops in the area of San Luis Potosi that could be used as food and feed resources with improved health benefits.

Nonmature seeds (developing seeds) were also collected as described before (*14*). Mature and nonmature seeds were harvested, cleaned, and ground using a mill with a 100 mesh. The flour was defatted with hexane at a flour/hexane ratio of 1:10 (w/v), and the resulting suspension stirred for 4 h at 4 °C. The slurries were then centrifuged at 13,000*g* for 20 min and the meal air-dried at room temperature. Defatted dry meals were kept in plastic bags at 4 °C until used. In order to investigate the effect of heating, popped amaranth seeds were prepared by heating the seed at 170 °C for 90 s in a traditional comal or hot plate (*15*). The flour obtained from popped amaranth seeds was also defatted. Commercial soybean seeds (*Glycine max (L.)* Merill), to be used as control material for lunasin extraction, were obtained from a local market and were milled and defatted following the same procedure as that previously described.

Protein Fractionation Procedure. The amaranth seed protein extraction was conducted according to Barba de la Rosa et al. (*16*), with some modifications. Extraction of albumin plus nonprotein nitrogen (NNP) fraction was carried out on defatted flour using distilled water as extracting agent. Suspensions of flour/extracting agent (1:10 w/v) were stirred for 30 min at 4 °C and centrifuged at 13000*g* for 20 min. The supernatant was collected and kept at −20 °C until use. The resulting pellet was used for 7S globulin extraction, resuspended in 0.1 M NaCl, 10 mM K_2HPO_4 at pH 7.5, and 1 mM EDTA, stirred, and centrifuged as mentioned above. The new pellet was used for 11S globulins, resuspended in 0.8 M NaCl, 10 mM K_2HPO_4 at pH 7.5, and 1 mM EDTA, stirred, and centrifuged as mentioned above. Prolamin fraction was extracted from the last pellet with 60% aqueous ethanol and glutelin fraction with 0.1 M NaOH.

Total protein extract was prepared using a Phosphate Buffered Saline (PBS) buffer at pH 7.4, in a ratio of 1:10 (w/v) flour/buffer. The material was extracted for 60 min using an ultrasonic bath, mixing every 10 min. The slurries were centrifuged at 13,000*g* for 20 min at 4 °C. The supernatants were collected and kept at −20 °C until use. All fractions were obtained in triplicate.

Quantification of Lunasin by ELISA. Indirect ELISA test was used for lunasin quantification as previously reported (*13*). A 100 μL of each protein fraction and total protein extract were placed onto the wells of Nunc Maxisorp 96-well plates, with high affinity for proteins. A standard curve of pure synthetic lunasin in distilled water was also run in a range of 24–72 ng/mL. Samples, standards and blanks were loaded in triplicate from three independent extractions. The plates were incubated for 14 h at 4 °C and then washed six times with 300 μL of the washing buffer (10 mM PBS and 50 mM Tween 20) at the lowest dispensing rate (150 μL/well/s) and aspiration rates (5 mm/s) to avoid protein detachment. The plates were then blocked for 1 h at room temperature by adding 300 μL per well of Tris-buffered saline (TBS; 50 mM Tris-HCl at pH 7.6 and 150 mM NaCl) plus 1% Tween 20 and 5% BSA. The plates were incubated with 50 μL of a 1:1000 dilution of lunasin mouse monoclonal antibody (TBS plus 3% BSA and 1% Tween 20), produced against the 21 amino acids from the carboxyl end of lunasin from soybean (provided by Dr. Ben de Lumen, University of California, Berkeley). After washing, the plates were incubated for 1 h with 50 μL of the antimouse IgG with the alkaline phosphatase conjugate using a 1:2000 dilution (Sigma Chemical Co, St. Louis, MO). The plates were washed again, and 100 μL of of *p*-nitrophenyl phosphate (pNPP) was added to each well to develop the color reaction during 1 h. The reaction was stopped with 2 N NaOH and the plates read at 450 nm in ELISA plate reader (Elx 808 IU from Biotek Instruments, Winooski, VT). Total protein concentration was measured using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). Lunasin concentration was expressed as μg lunasin equivalent/g of total protein.

Lunasin Identification by Western Blot. Total protein extracts and specific protein fractions of amaranth proteins were prepared at a concentration of 2 mg/mL, diluted 1:1 with tricine sample buffer, and boiled for 5 min. An aliquot of 20 μL of each sample was loaded onto 16.5% Tris-tricine polyacrilamide gels (Bio-Rad Laboratories, Hercules Inc., CA). Duplicates of gels were run in Mini Protean-3 Cells (Bio-Rad Laboratories, Hercules, CA) at 25 mA constant per gel for 180 min, using Tris-tricine-SDS buffer as the running buffer. One gel was fixed for 30 min in methanol/acetic acid/water (40:10:50 v/v/v) and stained for 1 h with Bio-Safe Coomassie Stain (Bio-Rad Laboratories, Hercules, CA). Destaining was done for 1 h with water and rinsed with fresh water for one additional hour. The pictures were taken with a Kodak Image station 440 CF. The second gel was directly soaked in 50 mL of transfer buffer (25 mM Tris base at pH 8.3, 192 mM glycine, and 10% v/v methanol) for 15 min. PVDF membranes were used for the blotting procedure. The transfer conditions were set at 300 mA (90 V) constant for 1.5 h, at 4 °C. After transfer, the membrane was rinsed twice for 15 min in TBS buffer (20 mM Tris and 500 mM NaCl at pH 7.5). The membrane was blocked for 1 h using 2% of ECL advanced blocking reagent (GE Healthcare Bio-Sciences Corp. Piscataway, NJ)

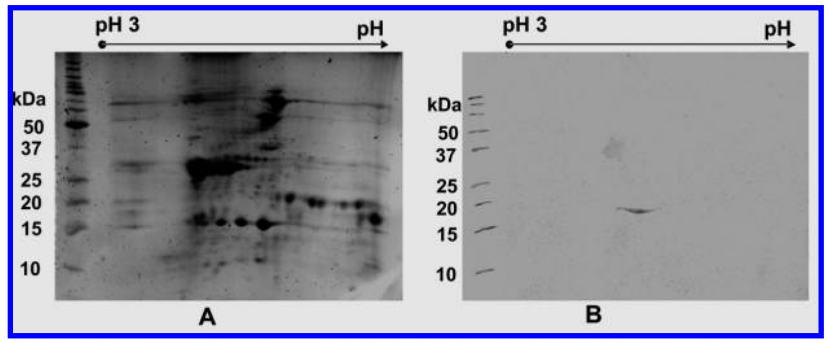

Figure 2. (**A**) Two-dimensional SDS−PAGE Coomassie blue staining of amaranth glutelins. (**B**) Western blot against lunasin antibodies.

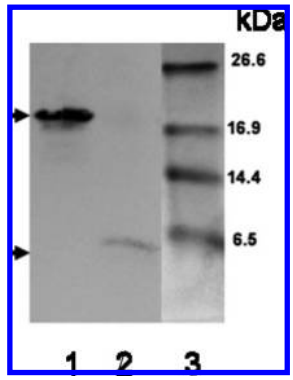

Figure 3. Western blot of immunoprecipitated lunasin-like protein from amaranth glutelins. Lane 1 = amaranth glutelin fraction; 2 = lunasin soybean enriched flour extract; 3 = molecular weight marker.

Table 2. MALDI-TOF Masses Analysis of Tryptic Digested 18.5 kDa Glutelin from Mature Seeds of Amaranth[a]

monoisotopic masses	sequence
655.991	HIMQK
1187.442	WQHQQDSCR
1312.620	WQHQQDSCRK
1327.504	QLQGVNLTPCEK

[a] The predicted sequences matched with those predicted sequences deduced from a tryptic cleavage of 5 kDa soy lunasin (sequence as follows: SK**WQHQQDSC RKQLQGVNLT PCEKHIMQK**I QGRGDDDDDD DDD).

plus 0.1% Tween 20 in TBS buffer and was washed twice with TTBS buffer (TBS buffer plus 0.1% Tween 20) for 15 min. It was then incubated overnight (12–14 h) at 4 °C with antibody buffer (2% ECL advanced blocking reagent in TTBS) containing the lunasin rabbit polyclonal antibody (in a 1:10,000 dilution), produced against the 21 amino acids from the carboxyl end of lunasin from soybean (provided by Dr. Ben de Lumen, University of California, Berkeley). After washing twice with TTBS for 15 min, the membrane was incubated with an antirabbit conjugated with the alkaline phosphatase (GE Healthcare Bio-Sciences Corp. Piscataway, NJ) secondary antibody, at 1:10000 dilution, and finally washed three times with TTBS following detection with the immuno-chemiluminescent substrate solution (GE Healthcare Bio-Sciences Corp. Piscataway, NJ), according to the manufacturer's instructions. The pictures were taken with a Kodak Image station 440 FC.

Two-Dimensional Protein Separation. Two-dimensional (2D) electrophoresis was performed according to the manufacturer's protocol (GE Healthcare Bio-Sciences Corp. Piscataway, NJ). The glutelin fraction was cleaned with the 2D cleanup kit (GE Healthcare Bio-Sciences Corp. Piscataway, NJ). The pellet obtained was solubilized with urea rehydration buffer (8 M urea, 2% CHAPS, 19 mM DTT, 0.5% immobilized pH gradient, IPG, buffer at pI 3–10, and 0.002% bromophenol blue). The concentration of protein was measured using Bradford assay. Isoelectric focusing (IEF) was performed at 20 °C according to the manufacturer's instructions (GE Healthcare Bio-Sciences Corp. Piscataway, NJ) loading 75 μg of protein onto 7 cm IPG strips using passive in gel rehydration. Prior to the second dimension, the IPG strips were first equilibrated for 15 min in 6 M urea; 30% glycerol; 2% SDS; 50 mM Tris-HCl at pH 8.8; and 1% DTT. The second dimension was done in 15% polyacrylamide gels. Duplicates of gels were run in Mini Protean-3 Cells at 25 mA constant per gel for 120 min. One gel was stained with Bio-Safe Coomassie Stain, and the second gel was used for Western blot analysis as mentioned before.

Immunoprecipitation of Lunasin. In order to purify lunasin from amaranth protein fractions, an immunoprecipitation assay was developed on the basis of the procedure reported by Bhat et al. (*17*). Briefly, for the preclearing step, 250 μL of protein fractions at a concentration of 2.5 mg/mL plus 2.5 μL of nonspecific rabbit IgG and 25 μL of protein A/G beads were added and mixed on an end-over-end mixer for 60 min at 4 °C; after that, the samples were centrifuged for 5 min at 10,000*g* at 4 °C. The supernatant was removed and placed in a new prechilled tube. After preclearing, 200 μL of each sample plus 300 μL of radio immunoprecipitation assay (RIPA) buffer were incubated with 2 μL of rabbit antilunasin polyclonal antibody for 60 min at 4 °C on the end-over-end mixer, and then 25 μL of protein A/G beads (Santa Cruz Inc. CA)were added and mixed on the end-over-end mixer overnight at 4 °C. After incubation with the antibody, the samples were centrifuged for 1 min at 10,000*g*. The pellet was washed 3 times with 500 μL of RIPA buffer and then resuspended in 100 μL of tricine sample loading buffer. The samples were boiled for 5 min and 20 μL loaded onto a gel. Duplicates of gels (16.5% polypeptides) were run in Mini Protean-3 Cells at 25 mA constant per gel for 180 min, using Tris-tricine-SDS buffer as running buffer. One gel was stained using the silver Quest Kit (Invitrogen, Carlsbad CA, USA) according to the manufacturer's instructions. The pictures were taken with a Kodak Image station 440 CF. The second gel was used for Western blot analysis.

Identification of Lunasin by MALDI-TOF. MALDI peptide mass mapping was conducted for confirming the identity of lunasin using an Applied Biosystems Voyager-DE STR at the protein facility core of the University of Illinois. The band that corresponded to the molecular weight identified by Western blot was cut from the gel of the immunoprecipitation assay.

The identity of lunasin was established by comparison of the peptide mass map of the putative peptide obtained from the gel tryptic digest with predicted masses of lunasin cleaved by trypsin. The peptides were extracted from the gel first with 50 mM ammonium bicarbonate and then with 50% (v/v) acetonitrile and 5% (v/v) formic acid (*18*). The digestion was performed with trypsin at a ratio 1:50 protein trypsin for 14 h. The analysis was done using MASCOT (Matrix Sciences, http://www.matrixcience.com/), the peptides were identified, automatically comparing with the NCBInr (http://www.ncbi.nih.gov) and Swiss-Prot databases (http://ca.expasy.org/sprot//).

Glutelin LC MS/MS Peptide *de novo* Identification. Glutelin fraction was digested with trypsin and analyzed by LC MS/MS for the search of *de novo* peptides with novel activities. For trypsin digestion, 200 μg of glutelin fraction was precipitated with acetone. The pellet was resuspended in 250 μL of urea buffer (6 M urea and 100 mM Tris at pH 7.4). The sample was reduced with DTT and alquilated with iodoacetamide. The digestion was done with trypsin at a ratio 1:50

Table 3. Biopeptides Identified in a Tryptic Digest of Amaranth Glutelin Fraction by LC/MS/MS

activity	ocurrence frecuency A	sequences with biological activity	description
enzyme inhibitor	0.0805	AP, FA, FP, GP, GPR, GQ, HA, IPI, KA, LA, LL, LP, MA, MP, PA, PP, PPLP, PPPA, VA, VP, VPL, VV	dipeptidyl-aminopeptidase IV inhibitor (*23*)
antyhypertensive	0.0566	AAP, AIP, ALPP, AVP, AY, FNQ, FP, FQP, FY, GGY, GKP, GRP, GY, HIR, HY, IKP, ILP, IR, IRA, IY, LAA, LAMA, LAY, LF, LLP, LNP, LPP, LQP, LQQ, LRP, LSP, LVL, LVR, LW, LY, MF, MY, PLP, PQR, PR, PRY, RF, RL*, RY, TAP, VAA, VAP, VAY, VF, VLP, VPP, VRP, VSP, VW, VY, VYP, YG, YGGY, YL, YP	angiotensin I converting enzyme inhibitor (*24*)
activating ubiquitin mediated proteolysis	0.0183	LA, RA, WA	activators of the ubiquitin-mediated proteolysis (*25*)
regulating	0.0123	DY, GFL, GLY, GP, LGY, PG, PGP	stimulate phagocytic cells implicated in the defense of the organism against infection (*26*)
antithrombotic	0.0095	PPG, PG, GP	involved in the homeostasis of the gastric mucosa and the anticoagulant and fibrinolitic potential of blood plasma (*27*)
antiamnestic	0.0093	PPG, PG, GP	potentate memory consolidation process in the central nervous system (*27*)
opioid	0.0062	NAGA, GYY, PLG, YG, YL, YPF	act as potent analgesics (*28*)
immunomodulating	0.0037	EAE, GFL, KRP, TKPR, YG, YGG	act as immunopotentiatores (*29*)
antioxidative	0.0036	HH, HL, LH, LHH	Offer protection against the peroxidation in cells (*30*)
ligand	0.0026	KK	act in the oligopeptide transport system (*31*)
neuropeptide	0.0010	KPS	showed antinociceptive activity (*32*)
immunostimulating	0.0009	KEEAE, LGY, LLY	stimulate phagocytic cells implicated in the defense of organism against infection (*33*)
embriotoxic	0.0005	RGD	shows embryotoxic activity (*34*)
anorectic	0.0004	PGP	inhibitor of insulin secretion (*27*)
anti inflammatory	0.0002	DTEAR	

Table 4. Effect of Tryptic Digest of Amaranth Glutelins on HeLa Cells in Comparison to that in Normal Cells

cells	treatment (μg/mL)	apoptotic cells (%)	cellular phase (%)		
			Go or G1	S	G_2
HeLa	control	3.2	60.7	15.5	20.6
	glutelins (1)	30.0	40.0	13.2	17.0
	glutelins (5)	38.8	42.2	9.0	10.0
	cisplatin (1)	70.0	ND[a]	ND	ND
fibroblasts	control	2.6	58.1	22.4	17.0
	glutelins (1)	2.8	57.6	15.9	24.1
	glutelins (5)	5.0	55.8	17.4	21.8
	cisplatin (1)	50.0	ND	ND	ND

[a] ND = not determined.

protein/trypsin for 14 h. The digestion was stopped by adjusting to pH 6 with concentrated acetic acid (*18*). LC-ESI MS/MS analysis was carried out on an LC-MS/MS system consisting of an Agilent 1200 series liquid chromatograph (Palo Alto, CA) coupled with an Applied Biosystems 3200 QTRAP tandem mass spectrometer (Foster City, CA) equipped with a Nano ion spray source at the protein facility core in CINVESTAV-IPN, México. Chromatography separation was done on an Agilent Zorbax 300SB C18 column (150 × 0.075, 3.5 μm), loading 10 μL of sample into the column. HPLC solvents were as follows: A, 98% (v/v) water, 2% (v/v) acetonitrile, and 0.1% (v/v) of formic acid; B, 98% (v/v) acetonitrile, 2% (v/v) water, and 0.1% (v/v) of formic acid. After a 30-min equilibration with 90% buffer A, the peptides were eluted directly into the electrospray source with a gradient from 60% to 80% of buffer B at a flow rate of 400 nL/min. Peptide identification was done using MASCOT (Matrix Sciences, http://www.matrixcience.com/). The identification of bioactive peptides was conducted by searching them in the active peptide database (http://www.uwm.edu.pl/biochemia).

Effect of Trypsin-Digested Glutelins, Lunasin-Containing Fraction, on Apoptosis and Cell Cycle Distribution of HeLa Cells. HeLa cells (immortalized cells from cervical cancer) were grown in Dulbecco's modified Eagle's media (DMEM) high in glucose supplemented with 5% fetal bovine serum, streptomicin (100 mg/L), penicillin G (62.1 mg/L), $NaHCO_3$ (3.7 mg/L), and amphotericin (1 mL/L), at 36 °C and 10% of CO_2. Apoptosis assay was performed by Tunnel (Tunel Labeling Kits, RnDSystems) and cell cycle distribution (*19*) using a FACS (fluorescence-activated cell sorting) apparatus (Calibur, Becton Dickinson, Franklin Lakes, NJ). HeLa cells with a confluence of 85% were treated with different amounts of trypsin-digested glutelins (1 and 5 μg/mL). Cisplatin (1 μg/mL) was used as a positive control of apoptosis. Primary culture of fibroblasts was used as the control of normal cells.

Model Projection of Bioactive Peptides in Amaranth. The search for bioactive peptides was conducted for all of the seed protein sequences reported for *Amaranthus* sp. at http://www.ncbi.nlm.nih.gov/entrez. Those sequences were then analyzed for the profile of active peptides using the database http://www.uwm.edu.pl/biochemia.

The occurrence frequency (A) of bioactive fragments with a particular activity in a polypeptide chain is given by the following equation:

$$A = \frac{a}{N}$$

where a = the number of amino acid residues forming fragments with given activity in protein sequence, and N = the number of amino acid residues of protein.

The frequency of occurrence of the identified active peptides in each amaranth protein was plotted on the *y*-axis, the proteins on the *x*-axis, and the activities on the *z*-axis (*20*).

Statistical Analysis. Tukey's test was used to determine significant differences among means. Trends were considered significant when the means differed at $p \leq 0.05$ (*21*).

RESULTS

Lunasin Concentration in Amaranth Seed Storage Proteins. The detection limit for soy lunasin using the ELISA method was 20.4 ng/mL. The corresponding standard curve was represented by the equation $y = 0.014x - 0.294$) (where y is

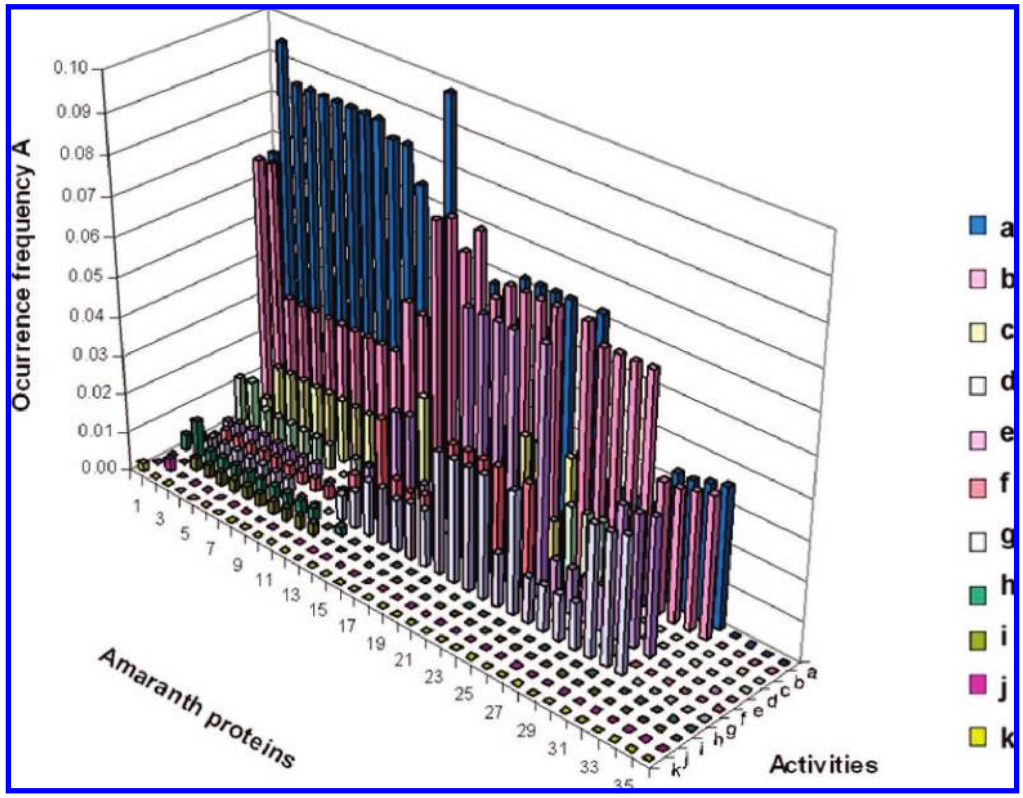

Figure 4. Predicted profiles of peptides in amaranth proteins with potential biological activities. The amino acid sequences were obtained from Swiss-Prot protein knowledgebase. The potential bioactive peptides and their functions were determined by searching the Biopep database (*20*). Amaranth sources: 1 = 11S globulin; 2 = antimicrobial protein; 3–8 = amaranth lectins; 9 = amaranth seed storage protein; 10 = seed specific protein of nutritional balance; albumins; 11 = lectin; 12 = antiviral/ribosome-inactivating protein; 13 = α-amylase; 14–17 = trypsin inhibitor; 18 = serine proteinase inhibitor; 19–22 = α-amylase; 23 = trypsin−chymotrypsin inhibitor serine protease; 24 = α-amylase; 25–27 = antimicrobial proteins; 28 = chitin binding AMPP 2; 29–31 = antimicrobial proteins; 32–36 = antiviral proteins. Activities: a = antihypertensive; b = protease inhibitor; c = opioid; d = activating ubiquitin-mediated proteolysis (AUMP); e = regulating; f = immunomodulating; g = antithrombotic; h = antioxidant; i = ligand; j = immunoestimulating; k = embryotoxic.

Globulin 11S sequence:

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1-50       sthasgffff hptkmakstn yfliscllfv lfngcmgegr frefqqgnec
51-100     qidrltalep tnriqaergl tevwdsneqe frcagvsvir rtiephglll
101-150    psftsapeli yieqgngitg mmipgcpety esgsqqfqgg ederireqgs
151-200    rkfgmrgdrf qdqhqkirhl regdifampa gvshwaynng dqplvavili
201-250    dtanhanqld knfptrfyla gkpqqehsge hqfsresrrg erntgnifrg
251-300    fetrllaesf gvseeiaqkl qaeqddrgni vrvqeglhvi kppsraweer
301-350    eqgsrgsryl pngveetics arlavnvddp skadvytpea grlttvnsfn
351-400    lpilrhlrls aakgvlyrna mmaphynlna hnimycvrgr griqivndqq
401-450    qsvfdeelsr gqlvvvpqnf aivkqafedg fewvsfktse namfqslagr
451-500    tsairslpid vvsniyqisr eeafglkfnr pettlfrssg qgeyrrkisi
501        a
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Figure 5. Amaranth globulin sequences reported at the GenBank database.

absorbance at 450 nm, and x is concentration in ng/mL) and $R^2 = 0.96$. As shown in **Table 1**, lunasin concentrations in total extracted protein from amaranth mature seeds ranged from 9.5 to 12.1 μg lunasin equivalent/g extracted protein, with no significant differences among cultivars ($p > 0.5$). Albumins, globulins, and prolamins showed lunasin concentrations (1.39 to 1.98 μg lunasin equivalent/g of protein) with no significant differences among cultivars ($p > 0.5$). Among protein fractions, glutelin showed the highest lunasin concentrations (2.71 to 3.01 μg lunasin equivalent/g of protein), significantly different from that of the other protein fractions ($p < 0.5$). Total lunasin concentration in amaranth was similar to that found in barley (5.9 to 8.7 μg/g of protein) where no significant differences were reported among cultivars studied (*11, 22*).

Lunasin Detection by Western blot. The polyacrylamide gel stained with Coomassie Brilliant Blue (**Figure 1A**), shows that amaranth proteins under denaturing conditions were present in the range from 10 to 200 kDa and that few proteins were present in the range of molecular weights below 7 kDa. By Western blot analysis, a band was detected with a molecular weight of 18.5 kDa, different from the one observed in soybean (line 9, 5 kDa). However, amaranth protein reacted immunologically to soy lunasin antibodies, and for this reason, it was considered as a lunasin-like protein. It was found in globulin 7S, globulin 11S, and glutelin fractions as well as in the total protein extract of mature seeds (**Figure 1B**, lanes 3–6). An additional band was also detected at the molecular weight of 15.4 kDa in the glutelin fraction (lane 5). Total protein extract

Table 5. Biopeptides Identified in Amaranth Globulins[a]

biopeptide	sequence	EC_{50}[b]	location[c]
β-lactokinin	RL	2439	[54–55], [254–255], [322–323], [342–343], [358–359]
	IR	695	[89–90], [145–146], [167–168], [454–455]
ACE inhibitor	GKP	352	[221–223]
	LF	349	[28–29], [31–32], [485–486]
	FP	315	[213–214]
	YL	122	[218–219], [309–310]
	RF	93	[40–41], [159–160], [216–217]
	HY	26	[375–376]
	VY	7	[335–336]
	VW	1	[73–74]
undefined	LLP	57	[99–101]
	FY	25	[217–218]
	AY	19	[186–187]

[a] The list of bioactive peptides identified is given as well as the exon locations. The validity of the predicted bioactive peptides is confirmed by using http://www.uwm.edu.pl/biochemia. [b] EC_{50}= effective concentration 50 μM. [c] Amaranth globulin sequence shown in **Figure 5**.

from nonmature seeds (lane 7) was tested, and it was found that even in early stages of seed development the lunasin-like band remained as 18.5 kDa. Popped amaranth (lane 8) showed the same band at 18.5 kDa as well. These data suggest that lunasin-like protein is not affected by extreme thermal treatments (170 °C in the center of the grain for 90 s) such as the one required to prepare popped amaranth (*15*).

Because of the fact that glutelin fraction showed the highest lunasin-like concentration (2.7–3.01 μg/g protein), this fraction was used to separate the peptides by 2D-PAGE and confirm its immunoreactivity by Western blot analysis. **Figure 2A** presents the Coomassie stained gel and **Figure 2B** the Western blot membrane. The membrane showed only one spot with a molecular weight of 18.5 kDa and p*I* 6.0, which is near the p*I* for lunasin in soybean.

Immunoprecipitation Assay and MALDI-TOF Characterization. The amaranth glutelin fraction was used to purify lunasin by immunoaffinity, using soybean-enriched flour extract as the control of the assay. As shown in **Figure 3**, only one band at 18.5 kDa was obtained. The 18.5 kDa was cut out, from the gel, for MALDI-TOF characterization. **Table 2** presents the masses of the in gel tryptic digestion of this band. The data show that the amino acid sequence of lunasin-like protein in amaranth overlaps by 60% with that of soy lunasin (bold letters).

Our laboratory is currently working on the detection of the cell adhesion motif as well as the eight aspartic acid residues at the carboxy terminal of the lunasin-like protein in amaranth.

Peptides Identified in Tryptic-Digested Amaranth Glutelin Fraction. LC/MS/MS analysis of the glutelin fraction digested with trypsin showed 508 *de novo* peptides. The profile of bioactive peptides (**Table 3**) showed that some of the activities were enzyme inhibition (*23*), antihypertensive activity (*24*), activating ubiquitin-mediated proteolysis (*25*), regulation (*26*), antithrombotic and antiamnestic activity (*27*) opioid activity (*28*), immunomodulating activity (*29*), antioxidant activity (*30*), ligand activity (*31*), neuropeptide activity (*32*), immunostimulating activity (*33*), embryotoxic activity (*34*), anorectic activity (*27*), and anti-inflammatory activity. Peptides such as AP, GPR, GQ, PPLP, and PPPA are involved in the inhibition of dipeptidyl aminopeptidase IV (*35*). The tripeptides LPP, LRP, and VPP as well the dipeptide YP have been shown to have ACE-inhibitory activity in spontaneously hypertensive rats (*24*). The tripeptides LRP and VPP, also found in sour milk, have been associated with hypotensive activity (*36, 37*).

Apoptosis and Cell Cycle Distribution of HeLa Cells Treated with Tryptic-Digested Amaranth Glutelin Fraction. As shown in **Table 4**, HeLa cells treated with tryptic-digested glutelin (1 μg/mL) caused 30% apoptosis, while fibroblasts only showed 2.8%. HeLa cells treated with cisplatin (1 μg/mL) showed 70% apoptosis and fibroblast cells showed 50% (**Table 4**). Increasing the glutelin concentration to 5 μg/mL revealed an increase to 38.8% apoptosis in HeLa cells and just 5.0% in fibroblast cells. The control, untreated cells, produced only an average of 3% apoptosis. These preliminary results have shown that the glutelin fraction has the ability to selectively cause apoptosis to neoplasic cells. The apoptosis phenomenon was observed in a period of 16 h. The effect of soy lunasin has been reported as the inhibition of core histone acetylation (*9, 11*). Further work must be done on amaranth lunasin and glutelin hydrolysates to determine their mechanism of action against HeLa cell proliferation.

Other Bioactive Peptides in Amaranth. Figure 4 presents information on the potential of bioactive peptides in amaranth proteins. There were 36 sequences for amaranth seed proteins reported in the database (*www.ncbi.nlm.nih.gov).* These sequences were tested for all of the 1573 active peptides reported (*www.uwm.edu.pl/biochemia)* with 39 different activities (*20*). Active peptides were found in amaranth proteins with 12 main activities: antiamnestic, antithrombotic, immunomodulating, opioid, regulating, antioxidant, ligand, activating ubiquitin-mediated proteolysis (AUMP), immunostimulating, embryotoxic, protease inhibiting, and antihypertensive (*38*). As can be seen in **Figure 4**, 11S globulin (amaranth protein 1) showed the highest frequency of antihypertensive activity (activity a).

From this bioinformatics analysis, we decided to analyze in more detail the amaranth globulin reported in the GenBank database with the accession No. X82121 (**Figure 5**).

Performing the same bioinformatics analysis, the location of the antihypertensive peptides was found in the globulin sequence (**Table 5**). Di and tripeptides as GKP, LF, YL, RF, and HY are reported in the literature as angiotensin-I-converting enzyme inhibitors (*24*).

DISCUSSION

This study shows that amaranth seed can be a potential source of various bioactive peptides with diverse bioactivities relevant to health, such as cancer and hypertension. Amaranth also contains a lunasin-like peptide, an anticancer bioactive compound, previously found only in soy (*13*), barley (*11*), and more recently in wheat (*12*). Lunasin has a relatively high concentration of aspartic acid (*39*). Amaranth glutelin fraction also has a relatively high concentration of aspartic acid, 10.6% of the total protein (*16*), suggesting that the acidic regions may be related to a lunasin epitope in the protein fractions of the seed.

The production of multiple defensive peptides from larger protein precursors may be widespread in plants (*11*). Lunasin is a peptide that could have a role in seed development, but the mechanisms are poorly understood (*40*). The lunasin-like protein could be part of a major protein. In plants, there are several mechanisms that regulate processes in seed development. This needs to be verified in the case of amaranth.

In the case of amaranth, the total protein extracts of both nonmature and popped seeds showed that the lunasin-like protein remains as a band at 18.5 kDa. The glutelin trypsin-digested fraction showed apoptotic activity against HeLa cells.

The *in silico* analysis revealed that the most frequent peptides in amaranth are antihypertensive, especially globulin 11S, which showed peptides with potential to inhibit the angiotensin-I-

converting enzyme (ACE). The ACE inhibitor plays an important role in the renin−angiotensin system, which regulates blood pressure. Inhibitors of this enzyme lower blood pressure, and many antihypertensive drugs are potent ACE inhibitors (*24*). In the glutelin fraction, structurally similar to the globulins but with different solubility (*41*), antihypertensive peptides were found by LC/MS/MS along with other interesting peptides with, for example, anorectic and anti-inflammatory activities. The glutelin fraction showed peptides that contain dipeptidyl aminopeptidase (DPP) IV inhibitors. DPP IV has been identified as a key enzyme responsible for the inactivation of gastric inhibitory polypeptide (GIP) and glucagons-like peptide-1 (*7*–*36*) amide (tGLP-1) that are important insulin-releasing hormones secreted from endocrine cells in the intestinal tract in response to feeding (*42*). There are reports in which a specific inhibitor of DPP IV lowered blood glucose and enhanced insulin secretion in glucose-treated diabetic obese Zucker rats, presumably by protecting against the catabolism of incretin hormones tGLP-1 and GIP (*35*). The design of potent and selective small molecule inhibitors of DPP IV has been suggested as a strategy for the treatment of impaired glucose tolerance and type 2 diabetes (*23*).

Since globulins and glutelins are the major fractions of amaranth seed storage proteins, amaranth can be considered as a natural potential source of antihypertensive peptides. For other seed storage proteins such as soybean globulin 7S or wheat α/β gliadin, the values of frequency for antihypertension activities ranged between 0.06 and 0.08, respectively, which are comparable to those found in this research for amaranth proteins. Furthermore, it is reported that potential biological activity is higher for proteins from seed sources than those from animal sources (*20*).

In conclusion, amaranth seed proteins could be an alternative source of lunasin or lunasin-like isoforms. In addition, amaranth seeds are a potential source of other bioactive peptides with biological functions that could be beneficial to health, particularly anti-hypertensive activity. This is the first study that reports the presence of a lunasin-like peptide and other potentially bioactive peptides in amaranth protein fractions. Work is currently underway in our laboratories to further evaluate their biological activity and impact on health.

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