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Characterization of Two Non-Fetuin-Binding Lectins from Tepary Bean (*Phaseolus acutifolius*) Seeds with Differential Cytotoxicity on Colon Cancer Cells

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Abstract

Two non-fetuin-binding lectins from tepary bean (*Phaseolus acutifolius*) seeds were purified and characterized. Both lectins LA and LB showed by two dimensional gel electrophoresis very similar apparent molecular mass, and their isoelectric point for both lectins were also very close. Mass spectrometry analysis confirmed these similarities showing high sequence homology among them, and when these sequences were compared to the Mirkov´s deduced sequence of the phyto-hemagglutinin from *Phaseolus acutifolius*, LA showed higher homology than LB. On the other hand, when their biological activity was determined, only LA possessed affinity to type A erythrocytes, and when these lectins using a semi pure fraction were measured for their cytotoxic effect on colon cancer cells, they showed a differential effect. In the semi pure protein fraction tested, the rest of no-lectin proteins separated by electrophoresis presented no activity. Our results indicate that despite the high homology in their sequence, isoelectric point, and apparent molecular mass, these two structurally closely related lectins, showed differences in their biological behaviour, represented by their agglutinatination activity, as well as, in their cytotoxic effect on human colon cancer cells.

Keywords: Lectins; Cancer; Glycoproteins; Tepary beans; *Phaseolus acutifolius*

Abbreviations: pI: Isoelectric Point; HPLC: High Performance Liquid Chromatography; MALDI: Matrix-Assisted Laser Desorption Ionization; FT-ICR: Fourier-Transform Ion Cyclotron Resonance; SEC: Size Exclusion Chromatography; FDR: Decoy False Discovery Rate

Introduction

Lectins are glycoproteins of non-immune origin which specifically and reversibly bind carbohydrates, producing agglutination of cells and precipitate polysaccharides and glycoproteins, due to the presence of at least one non-catalytic domain [1-3]. Although lectins are present in most living organisms, plant lectins is the most investigated group [4]. They can be classified into 12 families according to the structure of their carbohydrate-binding domains [5]. Plant lectins are mainly present in storage tissues such as seeds, representing up to 10% of the total protein, whereas in leaves, roots and stems they are present in lower concentrations [6-8]. The high concentration in storage tissues, their up-regulation by plant biotic and abiotic stress (inducible lectins), plus the results obtained on insects feed on artificial diets containing lectins (*in vivo* approaches), indicates that they are part of the defence mechanisms of plants [8-10]. Within the plant lectins, legume lectins possess a carbohydrate-binding domain, and also Ca^{2+} and Mn^{2+} atoms [4-11]. The potential of plant lectins to recognize altered glycosylation in carcinogenic tumors, as well as their adverse effects on cell growth of cancer cells *in vitro*, have been demonstrated [12-15]. Plant lectins have been used for the prognosis and diagnosis of cancer using lectin microarrays; when combined with antitumor drugs, plant lectins can lead to a synergistic effect for the treatment of breast cancer, while reducing the side effects [12,15,16].

Early reports described at least two different lectins present in tepary bean seeds; one with a high affinity for lymphocytes but poor affinity for erythrocytes and fetuin, and the second one with a high affinity for erythrocytes and fetuin and with low affinity for lymphocytes [17]. Recently, different properties of tepary bean lectins have been described [18-22]. A previous report by our group showed that a semi-pure fraction of tepary bean lectins with no affinity for fetuin, obtained by a combination of Size Exclusion Chromatography (SEC) and Ion Exchange Chromatography (IEC), differentially affected the survival of different cancer cells, particularly the colon cancer $CaCo_2$ cells and breast cancer MCF-7 cells. In addition, this fraction affected 3T3/v-mos transformed cell line proliferation in a dose-dependent manner with respect to their normal counterpart [23].

Although some tepary bean lectins have been purified and partially characterized using different methods, most of them were purified using fetuin affinity chromatography [20-23]. However, even in those studies, the presence of lectins that did not recognized fetuin was already mentioned. Among them, Castillo-Villanueva et al. [20] purified four tepary lectin isoforms using SEC and cationic exchange chromatography, and then analyzed by tandem Mass Spectrometry (MS/MS), revealing differences on their fragmented peptides. However, they all showed homology with the deduced tepary-lectin sequence reported by Mirkov et al. [24]. Similarly, the sequences of the two

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fetuin-binding lectin subunits purified by Valadez-Vega et al. [21] had also homology to the sequence reported by Mirkov and their pIs were also similar (4.2 to 4.5).

In this study, we identified the presence of two lectins with no affinity for fetuin, in the lectin fraction previously described by García Gasca et al. [23]. Despite their similarity in appMM, and Isoelectric Points (pI's), they showed differences in their biological properties.

Materials and Methods

Biological material

Tepary bean seeds were *Phaseolus acutifolius* var. *acutifolius* A. Gray.

Lectin purification and agglutinating activity determination

Lectins present in tepary bean seeds were extracted according to García-Gasca et al. [23]. Aqueous extracts were precipitated with ammonium sulfate ($[NH_4]_2SO_4$) according to Englard and Seifter [25]. The precipitaton was carried out eliminating first the protein fraction precipitated at 40% of ammonium sulfate saturation, and then collecting the fraction that precipitated at 60% ammonium sulfate saturation. This protein fraction was re-suspended, and dialyzed against deionized water. After dialysis, the precipitated fraction was passed through a G-75 Sephadex (Pharmacia Biotech, Uppsala, Sweden) gel filtration column (1.80 cm × 2.1 cm) equilibrated with 0.01 M ammonium bicarbonate (NH_4HCO_3), pH 7.8. Elution was completed at a flow rate of 0.3 mL min^{-1}, collecting 4 mL fractions. Those fractions showing agglutinating activity, against 2% glutaraldehyde-fixed rabbit erythrocytes were pooled and concentrated using an Amicon Ultrafiltration System (Millipore, Billerica, MA, USA) [26, 27]. This fraction was further purified by HPLC using a Zorbax GF-250 column (Agilent, Santa Clara, CA, USA) equilibrated with 0.01 M phosphate buffer (pH 7.0) with a flow rate of 1 mL/min. Peak fractions were concentrated by ultrafiltration. Fractions with agglutinating activity were evaluated by electrophoresis, and those containing a single band were used for protein characterization. Samples were always stored at 4°C.

Biological activity of LA, and LB

Hemagglutination activity of LA and LB, were tested against blood samples provided directly by known donors or by the Clinical Services Unit of the Faculty of Chemistry, Universidad Autónoma de Querétaro. Rh-positive types A, B and O erythrocytes were used. Briefly, the samples were washed with 1x PBS, trypsinized, bringing the volume to a concentration of 1% trypsin, fixed with 0.1% glutaraldehyde, and resuspended in 1x PBS for storage. LA, and LB were prepared at 1:1 dilution with 1x PBS, protein was quantified, and hemagglutination activity was calculated [25,27,28]. Each determination was done by duplicate with at least three independent experiments. Protein content was determined by the Bradford protein assay using Bovine Serum Albumin (BSA) as a standard [28].

Cytotoxic effect: HT-29 human colon cancer cells (ATCC, Manassas VA, USA) were seeded (3×10^4 cells per well) in 24-well plates with 10% fetal bovine serum (FBS) in DMEM (Gibco® BRL, Grand Island, NY, USA) supplemented with 62.1 mg/L penicillin G (Sigma-Aldrich; Cat. No. P-3032; St. Louis, MO, USA), 100 mg/L streptomycin (Sigma-Aldrich; Cat. No 15290-018), and 2.5 mg/L amphotericin b (Sigma-Aldrich; Cat. No A9528). The cells were kept at 37°C in a humidified atmosphere with 5% CO_2. After 24 h, the medium was changed to 2% FBS in the same medium, and 48 h after seeding, the cells from two wells were counted to determine the number of cells at the time of changing the experimental conditions (Co). Likewise, treatments were added in duplicate in DMEM supplemented with 0.5% BSA in concentrations between 0.001 to 2.6 mg of protein/mL. After 24 h incubation, the effects on cell proliferation and cell survival were determined [29]. Lineal regressions of log concentration against the rate of proliferation or survival were used to calculate the lethal concentrations (LC50) and the Inhibitory Concentrations at 50% (IC50). Each determination was done by duplicate with at least three independent experiments.

Electrophoretic separations and post electrophoretic carbohydrate determinations:

SDS-PAGE was performed using 10% resolving gels according to Schägger and von Jagow, Coomassie blue R-250 D was used for protein staining [30]. The electrophoresis (2DE) was carried out according to Görg et al. using a 7 cm immobilized pH gradient (IPG) strip with a linear pH gradient from pH 4 to 7 [31]. Glycoproteins present in the polyacrylamide gels were stained following the PAS technique [32].

Sample preparation prior for tandem Mass Spectrometry (MS/MS): the in-gel digestion protocol was based on the protocol reported by Shevchenko et al. [33] with modifications. After selecting the tepary bean lectin spots from the gels, samples were washed with deionized water, and 50%, and then 100% acetonitrile, for 30 min, 20 min and 10 min. The acrylamide gel samples were then incubated for 5 min at room temperature with 100 mM NH_4HCO_3. Next, 100% acetonitrile was added with stirring for 15 min at room temperature and the resulting supernatant was separated and dried using a Speed Vac device. Spots were reduced by incubating and shaking the sample for 45 min with 10 mM dithiothreitol (DTT) and 100 mM Tris-HCl, pH 9, at 56°C. After the incubation, the sample was cooled and dried.

Alkylation was accomplished by adding 55 mM Iodoacetic Acid (IAA) in 100 mM NH_4HCO_3, followed by incubation for 15 min in the dark. Next, the sample was washed and incubated for 15 min with 100 mM NH_4HCO_3 and 100% acetonitrile in the same proportions. The final sample was incubated for 15 min with 100% acetonitrile and was finally dried. To digest the samples, gels were rehydrated and then incubated for 45 min with trypsin (10 ng/µL) in 50 mM NH_4HCO_3 at 4°C. The supernatant was discarded, and the hydrated gel was incubated overnight with 50 mM NH_4HCO_3 at 37°C.

After digestion, the peptides were extracted and purified, eliminating the supernatant. The rehydrated gel samples were then incubated in water, sonicated, and centrifuged. The aqueous supernatant was recovered, and the process was repeated, this time using 60% acetonitrile/5% formic acid instead of water, and the obtained supernatant was added to the recovered sample. The mixture was dried, and 5% formic acid was added. The peptides were purified using ZipTip® C18 reverse-phase chromatography tips (Millipore, Bedford, MA, USA) according to the manufacturer protocols.

LC-MS\MS methods

LC separation was done on a Waters Nano Acquity (Waters) with a Proxeon nanospray source. The digested peptides were reconstituted in 2% acetonitrile/0.1% trifluoroacetic acid and loaded onto a 100 µ × 25 mm Magic C18 100 Å 5 U reverse phase trap where they were desalted online before being separated using a 75 µ × 150 mm Magic C18 200 Å 3 U reverse phase column. Peptides were eluted using a gradient of 0.1% formic acid (A) and 100% acetonitrile (B) with a flow rate of 300 nL/min. A 60 min gradient was ran with 5% to 35% B over 45 min, 35% to 80% B over 5 min, 80% B for 1 min, 80% to 5% B over 1 min, and finally held at 5% B for 8 min.

Mass spectra were collected on an LTQ-FT mass spectrometer (Thermo Fisher Scientific) in a data-dependent mode with one MS precursor scan followed by 10 MS/MS scans. Peptide fragmentation was performed using Collision Induced Dissociation (CID) with a Normalized Collision Energy (NCE) value of 30. Unassigned charge states as well as +1 and ions >+5 were excluded from MS/MS fragmentation.

Database searching: tandem mass spectra were extracted and charge state deconvoluted by Proteome Discover 2.2. Deisotoping was not performed. All MS/MS samples were analyzed using X! Tandem (The GPM, thegpm.org; version CYCLONE (2013.02.01.1)). X! Tandem was set up to search all Phaseolus proteins in the NCBInr database plus an equal number of decoy sequences (144500 entries) assuming the digestion enzyme trypsin. X! Tandem was searched with a fragment ion mass tolerance of 0.40 Da and a parent ion tolerance of 20 PPM. Carbamidomethyl of cysteine was specified in X! Tandem as a fixed modification. Glu->pyro-Glu of the n-terminus, ammonia-loss of the n-terminus, gln->pyro-Glu of the n-terminus, deamidated of asparagine and glutamine, oxidation of methionine and tryptophan, dioxidation of methionine and tryptophan and acetyl of the n-terminus were specified in X! Tandem as variable modifications.

Criteria for protein identification: scaffold (version Scaffold_4.4.6, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 98.0% probability to achieve an FDR less than 1.0% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 96.0% probability to achieve an FDR less than 5.0% and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm [34]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

Results

After purification of the tepary bean seed proteins by SEC, two main protein peaks were obtained LA and LB, each one with the presence of two main glycoproteins (Figure 1).

To determine their biological activity, the hemagglutination of type A, B, and O, Rh-positive human erythrocytes was assayed. Only LA possessed affinity to type A erythrocytes, while LB showed no difference in recognition (Figure 2).

Cytotoxicity on cancer cells was assayed (Figure 3) and similar results were found for both fractions, however, LB showed a higher antiproliferative effect (IC50 166 µg protein/mL, y=-25.989x+29.697,

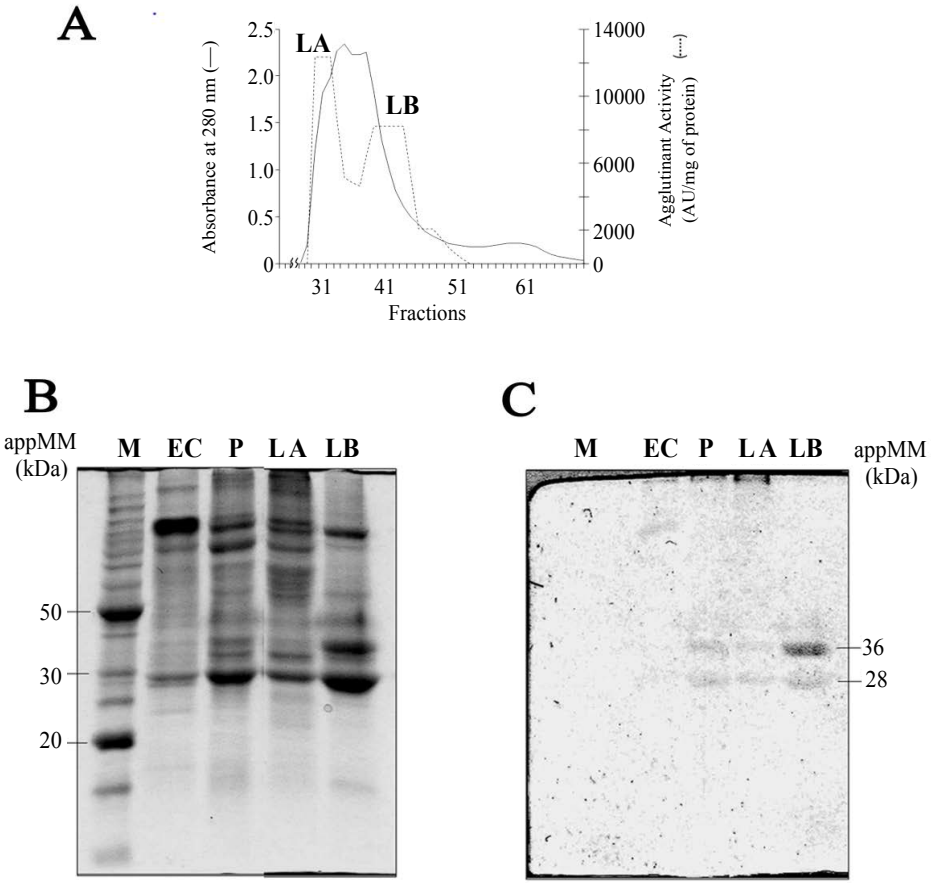

Figure 1: Purification profiles of the two main agglutinant activity-enriched fractions of Tepary bean proteins [A: Size Exclusion Chromatography (SEC) of Tepary bean proteins precipitated with ammonium sulfate. B: SDS-PAGE of Tepary bean proteins of the first steps of purification. C: Glycoprotein profile of B. M: molecular mass markers. EC: crude extract. P: precipitated proteins with ammonium sulfate. appMM: apparent molecular mass. LA and LB: SEC-purified agglutinating activity peaks].

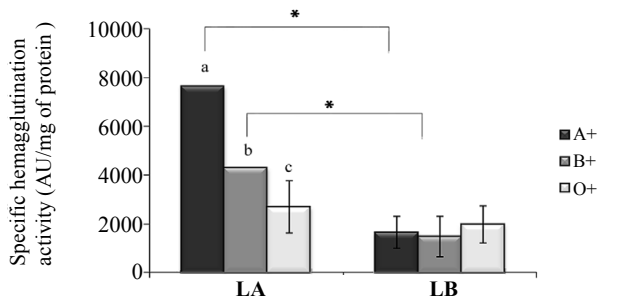

Figure 2: Comparison of the binding activity of the LA and LB on human erythrocytes of different blood types. Lowercase letters indicate statistically significant differences for blood types for each sample (Tukey, p<0.05). * Indicates statistically significant differences between samples for the same blood type (T student test, p<0.05). Each determination was done by duplicate with at least three independent experiments.

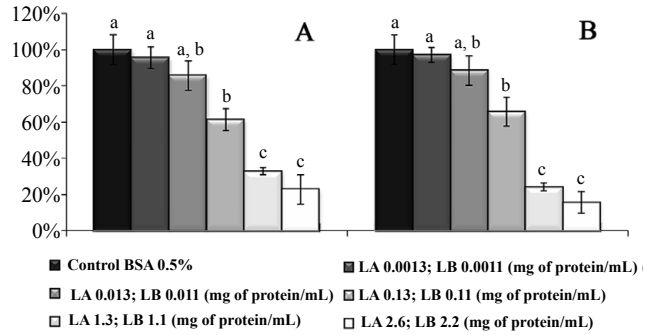

Figure 3: Concentration-response curves for LA (A) and LB (B) on HT-29 cells proliferation. Dose-responses curves were performed with concentrations between 0.001 to 3 mg of protein/mL for LA or LB. IC50 (256 and 166 µg of protein/mL for LA and LB, respectively) values were calculated by linear regressions of the concentration log *vs.* proliferation percentage. Lowercase letters indicate statistically significant differences for blood types for each sample (Tukey, p<0.05). Each determination was done by duplicate with at least three independent experiments.

R2=0.94) than LA (IC50 256 µg protein/mL, y=-22.865x+36.465, R2=0.97). The cytotoxic effect was not related to the hemagglutination activity. These results agree with our previous results and with those of Valadez-Vega et al. [21] because all the assayed fractions affected cell proliferation, and were dose-dependent [4,22]. Although the final effect was highly specific to the cell line studied, and the IC50 values were within the same order of magnitude. However, the lectins reported in our study differ from the previously reported in that these lectins did not bound fetuin.

Both LA and LB peaks were further purified by HPLC using a size exclusion column. In both purification steps, only one 28 kDa-lectin was detected using 2DE and the Schiff/periodic acid, carbohydrate staining technique (PAS) (Figure 4). These lectins showed very similar pIs (4.6 for LA, and 4.7 for LB).

Then, LA and LB were eluted directly from the 2DE gel, trypsinized, and subject to nano LC-MS/MS analysis. They produced 1,636 spectra (at 98.0% minimum, 0.32% spectra decoy False Discovery Rate (FDR), and 21 proteins (at 96.0% minimum, 1 min # peptides, 5.0% protein decoy FDR). The mass spectra obtained from LA and LB lectins showed high identity with phytohemagglutinins and other Phaseolus genus proteins (Table 1). The detailed record of the homologous proteins of LA and LB mass spectra are summarized in the protein report supplementary data. A high percentage of identity with the phytohemagglutinin of the *Phaseolus acutifolius* deduced sequence reported by Mirkov et al. [24] was found. In this regard, the mass spectra analysis of the peptides obtained from LA produced 54 exclusive unique peptides, 83 exclusive unique spectra, 498 total spectra, and 191/276 amino acids (69% coverage). The results of the mass spectra analysis of the peptides obtained from LB were: 27 exclusive unique peptides, 40 exclusive unique spectra, 618 total spectra, and 160/276 amino acids (58% coverage). The sequence coverage of LA and LB with the tepary bean lectin deduced sequence is shown at Figure [24]. The detailed record of peptide identification from LA and LB mass spectra are summarized in the peptide report supplementary data.

Homologue protein name	Protein molecular weight (Da)	Percentage sequence coverage	
		LA	LB
gi|500745|gb|AAA82181.1| phytohemagglutinin [*Phaseolus acutifolius*]	29,756.20	69.20%	58.00%
1|dbj|BAB72258.1| alpha-amylase inhibiotr AI-Pa1L [*Phaseolus acutifolius*]	31,091.50	45.60%	41.60%
gi|19744134|emb|CAD28674.1| phytohemagglutinin [*Phaseolus vulgaris*]	29,516.80	34.10%	29.30%
gi|19773406|emb|CAD29133.1| lectin [*Phaseolus vulgaris*]	29,570.40	30.50%	21.50%
gi|609411906|pdb|3WCR|A Chain A, Crystal Structure Of Plant Lectin (ligand-free Form)	27,599.70	29.10%	26.80%
gi|130011|sp|P15231.1|PHAM_PHAVU RecName: Full=Leucoagglutinating phytohemagglutinin; Short=PHA-L; Flags: Precursor	29,421.50	28.90%	30.80%
gi|312982406|gb|ADR30064.1| legumin [*Phaseolus vulgaris*]	70,802.50	26.20%	9.41%
gi|54019693|emb|CAH60212.1| phytohemagglutinin-L precursor [*Phaseolus costaricensis*]	29,404.40	24.90%	---
gi|561026078|gb|ESW24763.1| hypothetical protein PHAVU_004G158000g [*Phaseolus vulgaris*]	29,974.90	24.20%	---
gi|130010|sp|P05087.1|PHAL_PHAVU RecName: Full=Leucoagglutinating phytohemagglutinin; Short=PHA-L; Flags: Precursor	29,556.60	21.30%	16.20%
gi|54019703|emb|CAH60217.1| lectin precursor [*Phaseolus glabellus*]	29,862.70	19.10%	---
gi|54019697|emb|CAH60214.1| lectin precursor [*Phaseolus leptostachyus*]	29,741.10	16.20%	16.50%
gi|561017930|gb|ESW16734.1| hypothetical protein PHAVU_007G180800g [*Phaseolus vulgaris*]	38,846.30	12.40%	10.70%
gi|608601927|gb|AHW49428.1| alpha-phaseolin [*Phaseolus vulgaris*]	48,621.90	7.44%	---
gi|561027465|gb|ESW26105.1| hypothetical protein PHAVU_003G091300g [*Phaseolus vulgaris*]	31,787.90	7.14%	16.70%
gi|561012405|gb|ESW11266.1| hypothetical protein PHAVU_008G015400g [*Phaseolus vulgaris*]	61,908.60	7.10%	4.01%
gi|561011458|gb|ESW10365.1| hypothetical protein PHAVU_009G202900g [*Phaseolus vulgaris*]	49,219.50	5.09%	---
gi|56237708|emb|CAI26294.1| alpha-amylase inhibitor-like precursor [*Phaseolus maculatus*]	27,941.90	3.89%	---
gi|561004965|gb|ESW03959.1| hypothetical protein PHAVU_011G055500g [*Phaseolus vulgaris*]	67,511.60	---	3.91%

Table 1: Homolog proteins resulted from the MS/MS analysis of the spectra obtained from fragmentation of tepary bean lectins LA and LB.

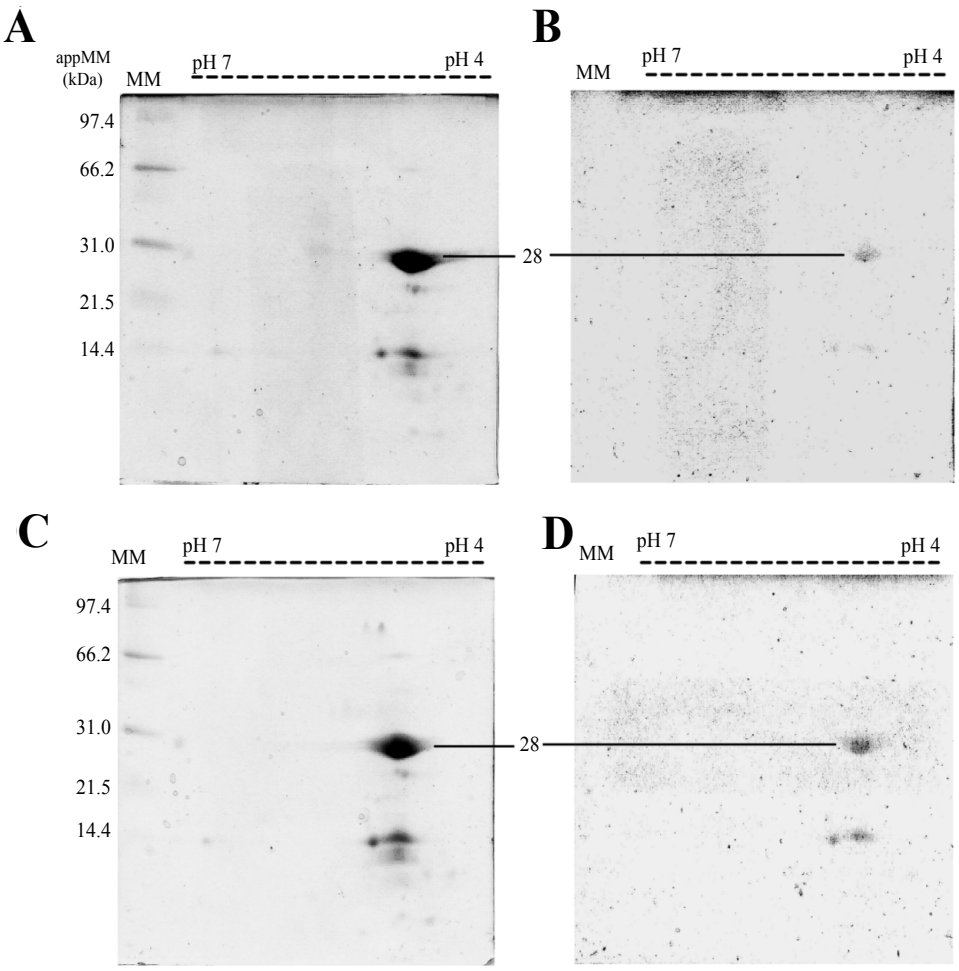

Figure 4: Bi-dimensional electrophoresis (2DE) of LA and LB. A: protein pattern of LA. B: glycoprotein pattern of LA. C: protein pattern of LB. D: glycoprotein pattern of C. MM: molecular mass markers. appMM: Apparent Molecular Mass. The isoelectric point (pI) separation was done from pH 4 to 7, and it is indicated as a dashed line over each gel.

Sequences of regions of the tepary lectin deduced protein (Mirkov et al. [24])		Lectin that match on that sequences						
		LA	LB	Li■	Lii■	Liii■	Liv■	Lv▲
25-34	ANDISFNFQR	■	■					
37-39	ETN		■					
40-55	LILQGDASVSSSGQLR	■	■					
56-72	LTNLNDNGEPTLSSLGR	■	■	■	■	■	■	■
91-101	SFATSFTFNIR	■						
102-124	VPNNAGPADGLAFALVPVGSKPK	■	■			■	■	■
125-130	DRGGLL	■	■					
131-135	GLFDG	■						
140-153	AHTVAVEFDTLYNR	■	■	■	■	■	■	■
154-158	DWDPR	■	■		■		■	
159-160	ER	■	■					
161-170	HIGIDVNSIK	■	■	■	■		■	■
171-173	SIK	■	■					
195-196	TK	■						
197-208	LLVASLVYPSQK	■	■					■
209-220	TSFIVSDTVDLK	■	■					■
235-239	SGITK	■						
240-255	GNVETNDLLSWSFASK	■	■		■	■	■	
256-266	LSDGTTSEGLN	■	■	■	■	■	■	
267-276	LANFVLNQIL	■		■	■	■	■	

Table 2: Comparison of the MS/MS-derived peptide sequences from LA and LB with other reported tepary bean lectin sequences (■ Tepary bean isolectins purified by Castillo-Villanueva et al. [20]; ▲ Tepary bean lectin sub-units purified by Valadez-Vega et al. [21]).

Discussion

Tepary bean seeds contained a variety of lectins that may participate in the defence of the plant, and that may also have pharmaceutical and biochemical potential for their use against different types of cancer cells, as well as in the early detection of cancers. However, to make use of these properties, it is necessary to know more about their structures. We established the amino acid sequences of the two protein fractions (A and B), which render a larger number of peptide sequence that other forms previously reported (Table 2), and we showed that these two non-fetuin-binding lectins from tepary seeds, regardless of their high sequence homology determined by mass spectrometry, their high similarity in appMM, and their pI, yet they showed differences in their specific agglutination activity, as well as in their differential cytotoxic effects on colon cancer cells. Analyses of the fragmented peptides from the MS/MS of LA and LB showed LA to be the closest lectin to the Mirkov-deduced sequence, with 70% of sequence coverage, whereas LB had 58%. Comparing these results with other tepary lectins previously reported, LA and LB have the closest sequence coverage to the Mirkov's tepary bean sequence (Table 2). In addition, two common and highly conserved amino acid sequences found in all analyzed tepary bean lectins were identified: amino acids 56–72 (LTNLNDNGEPTLSSLGR) and 140–153 (AHTVAVEFDTLYNR). These fractions also showed a high identity with *Phaseolus vulgaris* phytohemagglutinins and less identity with other legume lectins (data not shown). LA and LB had identical C-terminal regions (amino acids 240–276) with the four erythrocyte-binding isolectins reported by Castillo-Villanueva et al. [20] (Table 2).

These results indicate that small changes in the structure of the molecules can produce changes in their biological activities. Therefore, it would be useful to study the structures of the different lectins that have been reported from tepary bean, to shade some light on the structure-function relationship for these lectins.

Supporting Information

Tandem MS/MS Peptide and Protein Reports Supplemetary data (XLS).

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