

Original Article

PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF A T/TN SPECIFIC LECTIN FROM *LEPECHINIA BULLATA* SEEDS (LAMIACEAE)

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ABSTRACT

Objective: This study focused on purifying and characterizing a lectin from *Lepechinia bullata* (*L. bullata*) seeds, and determining its specificity towards tumour-associated carbohydrate-antigens.

Methods: Pigments were removed by washing the seeds with NH₄OH 0.1 M pH 9.4 and treating the crude extracts with Pectinex®. The purification procedure consisted of anion exchange chromatography on diethylaminoethyl (DEAE)-Sephadex followed by affinity chromatography. For the characterization, the phase was used polyacrylamide gel electrophoresis-sodium dodecyl sulphate (SDS-PAGE), isoelectric focusing, hemagglutination assays, enzyme-linked lectinosorbent assay (ELLA) and thermal shift assay (TSA).

Results: 6.2 mg of lectin were obtained from 100 g of seeds. It was able to agglutinate enzymatically treated erythrocytes with a minimal required lectin concentration of 7 µg. ml⁻¹. Strong binding to asialo bovine submaxillary mucine (aBSM) was determined, corroborating Tn recognition.

The isoelectric focusing showed a unique band at pH 8.5. Lectin pure shown bands at 28, 48 and 93 kDa by SDS-PAGE, with an incomplete dissociation of the last species despite trying several reduction conditions. By preparative electrophoresis under different conditions, three species were observed too, in all fractions one band at 28 kDa on Tricine-PAGE in reducing and no reducing conditions were found.

Amino acid composition, carbohydrate content, thermal stability and Ca²⁺ and Mn²⁺ requirements were determined. N-acetylgalactosamine (GalNAc) and desialylated mucins inhibited the agglutinant activity on human cells. Fetuin inhibited hemagglutination of rabbit erythrocytes.

Conclusion: A new lectin was isolated and characterized from *L. bullata* seeds, it recognizes T/Tn antigen and shows some similarities with other Lamiaceae lectins.

Keywords: Lamiaceae lectin, *Lepechinia bullata*, Protein, Purification, T/Tn antigen

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INTRODUCTION

Lectins are widely distributed in nature where they can be found in viruses, bacteria, plants and animals. Those specific carbohydrate binding proteins are different from sugar-specific antibodies and enzymes as well as from sugar transport proteins [1]. Lectins present high potential as diagnostic tools for diseases associated with alterations in cellular glycosylation, as found in various cancers [2]. Several studies have been focused on the search for the tumor markers and their therapeutic alternatives. A special interest has given to the Thomsen-Friedenreich antigen (TF or T, galactose (Gal) β1,3 GalNAcα-O-serine (Ser)/threonine (Thr)) as well as to its precursor, the Tn antigen (GalNAcα-O-Ser/Thr) and its sialylated forms (sTn), which are normally cryptic structures in the peptide core of O-glycoproteins [3–8]. The expression of T, Tn and sTn antigens is associated with several carcinomas and allows, in some cases, the prognosis of the clinical course [3, 4]. The importance of the Tn antigen and its expression in other disorders and diseases has recently been reviewed [6]. Thus, lectins able to recognize these structures are highly sought for in particular in plants [9]. However, Lamiaceae lectins have been little studied despite preliminary reports on their ability to recognize the Tn antigen [10–12].

Detailed studies have been carried out on a few Lamiaceae species from the Northern hemisphere's temperate zone until now [13–17] and the lectin from *Salvia sclarea* seeds (SSL) was the first to be isolated and partially characterized [14]. By contrast, species from the neotropical have been little explored despite their great diversity. A systematic survey has been conducted on species

belonging to the neotropical subgenus *Calosphace* [18, 19] and certain species naturalized in the New World have also been investigated [20], some having commercial value. Given the abundance of Lamiaceae species in Colombia and the potential clinical applications [21], our group undertook a systematic search for the identification, isolation, and characterization of lectins from selected species with the determination of their biological activities [20, 22]. The lectins from *Salvia patifolia* Kunth and *Hyptis mutabilis* (Rich.) Briq [23]. Have been partially characterized and a detailed work has been done with *Salvia bogotensis* Benth. lectin (SBoL) [24, 25]. The biochemical characterization of SBoL showed some differences with SSL although both lectins recognize specifically the Tn antigen.

Lamiaceae lectins isolated from nutlets (seeds) have shown some common features at the level of their molecular properties. They appear as dimers/tetramers close to neutral pH. It is worth to notice that *Moluccella laevis* lectin (MLL) possess a unique structural arrangement, as the 67, 42 and 26 kDa subunits are present in the absence of reducing agents and only the 26 kDa subunit is active after affinity chromatography in the presence of 8 M urea [26]. They show a relatively high carbohydrate content and the structure of the N-linked oligosaccharide (2-3 glycan/polypeptide chain) has been elucidated for SSL [16]. Preliminary evidence indicate a similar structure for the MLL oligosaccharide [27]; the analogous structure is also suggested in SBoL [25] which presents 3-4 oligosaccharide units per monomer. All characterized Lamiaceae lectins are basic proteins as determined by their experimental isoelectric point (pI)

values. GalNAc is the most potent inhibitor amongst monosaccharides and they show high affinity for asialomucins such as asialo ovine submaxillary mucin (aOSM) or aBSM, which are the best erythroagglutinin inhibitors. Thus, those have been used efficiently as ligands in affinity chromatography.

Hitherto, the *Vicia villosa* isolectin B4 (VVB₄) [28] from leguminosae family is the most specific one for detecting Tn antigen, this could hamper its use as a glycobiochemistry tool. Limited structural information is available for lamiaceae lectins since their purification is difficult and does not give the substantial amount of pure protein necessary for its tertiary structure determination. Likewise, their sequence in nucleotides or amino acid is generally unknown which prevent their production in recombinant form.

In order to deepen the knowledge of Lamiaceae lectins, we chose the *L. bullata* species for this study, taking into account its endemic character, the availability of substantial amounts of seeds and its high lectin activity (85%). The neotropical genus *Lepechinia* Wild. has 10 taxa in Colombia [20]. The objective of this work was to isolate and characterize *L. bullata* lectin (LBL) prior to the investigation on its interaction with Tn-bearing cells.

MATERIALS AND METHODS

Chemicals and vegetal material

The reference voucher of plant *L. bullata* species, from which seeds were collected (herbarium collection: COLO. Boyacá. Arcabuco-Gachantivá-JLF-557667 and COLO. Boyacá. Arcabuco-Gachantivá-JLF-557668) were deposited in the colombian national herbarium (COL). These correspond to the locality: outside road, Km 1.5 (N 05 °46.265'. W073 °28.266'. 2584 meters above sea level (MASL) and 4.5 (N 05 °46.250'. W073 °28.244'. 2600 MASL, Arcabuco-Gachantivá (Boyacá, Colombia). Fresh human blood was obtained from the clinical laboratory of Universidad Nacional de Colombia, Boyacá. Animal erythrocytes were supplied by the haematology laboratory of the Veterinary Faculty (Universidad Nacional de Colombia, Boyacá). Rabbit red blood cells were obtained from BioMérieux Laboratories. BioRad equipment was used for chromatography and electrophoresis. Fetuin-Agarose and proteins used as standards were all from Sigma-Aldrich. Bovine submaxillary mucin (BSM) and ovine submaxillary mucin (OSM) were from Fluka. Sugars were commercial products having the highest available purity. The rest of reagents were analytical grade. Enzymes (neuraminidase, β -galactosidase, papain and trypsin) used for erythrocytes degradation were from Sigma-Aldrich.

General methods

Mature *L. bullata* seeds were grinded to a fine powder. With the resulting flour, non-protein nitrogen content was determined, according to Pérez *et al.* [29]. Carbohydrate inhibition, demetallization assays, neutral sugar and amino acid determination were performed according to the methods described in Pérez [30]. Protein quantification was carried out using the micro Kjeldahl method or bicinchoninic acid (BCA) assay [31, 32]. In demetallization assay, the lectin was dialyzed first against 50 mmol ethylenediamine-tetraacetic acids (EDTA) and then against phosphate buffer saline (PBS) pH 7.3 \pm 1.0.

Lectin extraction

Several procedures were assayed to remove pigments or to diminish their formation due to the high polyphenol content in seeds. The final procedure was as follows. In small-scale experiments, 1.0 g seeds were left to soak in 10 ml NH₄OH 0.1 M pH 9.4 for 1 h with continuous stirring at room temperature (RT), then, the seeds were dried at RT and left to soak in PBS 20mmol mmol NaCl-5 mmol thiourea, pH 7.3 \pm 1.0 [33] for 2-3 h, at 4 °C and were then macerated and extracted in PBS-5mmol thiourea with continuous stirring at 4 °C, for 8 h. The extract, which had a viscous appearance, was centrifuged (38 000 gravities (xg), 1 h, 4 °C). The supernatant was recovered whilst this extraction procedure was reapplied three times on the pellet. Protein quantity and haemagglutination activity were determined immediately. The first extract was also treated with polygalacturonases mix (Pectinex®) due to high viscosity as described below.

Larger scale extraction (50 g) was done in PBS-5 mmol thiourea buffer with four successive extractions steps as described above. An aliquot (10-20 ml) of each extract was taken for nitrogen determination by micro-Kjeldahl. The extracted pool was extensively dialyzed against PBS-5 mmol thiourea buffer and tested for haemagglutination and lectin specificity was initially studied by ELLA.

Pectinex® treatment

Extract pH was adjusted to pH 4.7 with 200 mmol acetate buffer. Pectinex® Ultra SP-L (Novo) was then added (40 μ l/3 ml extract); the solution was incubated overnight at 37 \pm 0.5 °C. The extract was centrifuged (38 000 xg, 1 h, 4 °C), the supernatant was dialyzed against PBS pH 7.0 and lectin activity was determined. Control of experiments was made through dialysis and SDS-PAGE and no evidence of proteolytic activity in Pectinex® was shown.

LBL purification

Protein purification was carried out using both ionic exchange chromatography (IEC) and affinity chromatography. Briefly, extracts were passed through a column (2 x 15 cm) with DEAE-Sephadex resin (GE Healthcare) equilibrated in PBS. The non-retained peak (peak I) was obtained with PBS and was later used for affinity chromatography after concentration on Amicon® PM 10. Different affinity chromatography experimental conditions were assayed leading us to the following method: aBSM was prepared by digesting 50 mg BSM (Sigma-Aldrich) in 0.01 N HCl, 80 °C, 1 h; freeze-drying removed acid. The affinity supports were prepared by coupling aBSM to Sepharose 4B following the general techniques [34], by using activated sepharose 4B (Sigma-Aldrich), cyanogen bromide (CNBr), N-hydroxysuccinimide (NHS) and 6-aminohexanoic acid (ECH) (GE Healthcare) preactivated, following manufacturer specifications.

Lectin-active fractions coming off ultrafiltration were applied to the aBSM-Sepharose 4B column (2 x 19 cm) equilibrated with PBS. The resin was washed with PBS prior elution with 50 mmol tris (hydroxymethyl) aminomethane (Tris)-OH pH 11.4. 100 μ l HCl 2 N were added to the 1.0 ml fractions to obtain a final pH close to neutrality. Fractions were pooled and extensively dialyzed against 20 mmol NH₄HCO₃ prior freeze-drying. Protein concentration, specific agglutination activity (SAA) and ELLA assays were carried out using PBS-dissolved protein. The resin's retention capacity was assessed by applying increasing amounts of pooled extracts and checking the presence of lectin in the flow-through.

Polyacrylamide gel electrophoresis

SDS-PAGE was performed in non-reducing and reducing conditions (dithiothreitol (DTT) (40-100mmol), heating 100 °C, 10 min) on a 12.5% separating gel (12.5% T, 2.7% C) according to Laemmli [35] or Schägger and Von Jagow [36], using 10-20 μ g protein/well. As a molecular weight standard Opti-protein XL Marker® (ABM) was used, which has a protein range of 245 to 11 kDa; Precision Plus Protein Standards Unstained® (Bio-Rad), with a range between 250 and 20 kDa; or a protein mixture of bovine serum albumin (BSA) (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), β -lactoglobulin (18 kDa) and lysozyme (14 kDa). The run was performed at 100 volts for 1.5 h. Gels were stained using Coomassie G-250 or Instant blue (Expedeon) and later analyzed with the VersaDoc® or Gel-Doc imager systems (Bio-Rad).

Molecular weight

Subunit relative molecular weight (Mr) was determined by PAGE using Laemmli procedure [35]. 10 μ g of LBL were runned under reducing and non-reducing conditions. Native protein molecular weight was determined by preparative electrophoresis carried out according to the procedure described by Sánchez [37].

Lectin detection assay

Lectin activity was determined on crude extracts treated or non-treated with Pectinex® and on purified protein by ELLA according to the previously described procedure [22, 38, 39]. 96 well plates (NUNC, F16 Maxisorp) were sensitized with aBSM (0.14 μ g \cdot ml⁻¹) and biotinylated VVB₄ (1 mg \cdot ml⁻¹) was used as a specific control.

Streptavidine-peroxidase (1.3 $\mu\text{g}\cdot\text{ml}^{-1}$) and H_2O_2 , 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) were added after suitable washes and absorbance at 415 nm were read on a plate autoreader (Bio-Rad). The activity (%) was calculated as

$$\% = 100 - \left(\frac{\text{Sample absorbance}}{\text{Control absorbance}} \times 100 \right)$$

Hemagglutination assay

Assays were done on human and animal red blood cells (RBCs) as described in Pérez [30]. For A+human erythrocytes, the treatment described by Hirohashi et al. was used [40], RBCs were enzymatically treated to expose T or Tn determinants using neuraminidase and β -galactosidase stock at a concentration of 0.5 $\text{mg}\cdot\text{ml}^{-1}$ in 1X PBS. Hemagglutinating activity was tested in presence and absence of 5-100 mmol Ca^{2+} and Mn^{2+} (chloride ions). The effect of temperature was assessed after incubating the lectin at 50 $\mu\text{g}\cdot\text{ml}^{-1}$ in PBS for 1 h between 25-92 °C. SAA was determined as being the minimal protein dilution required for agglutination.

For rabbit RBCs, a 4% erythrocytes solution (bioMérieux, France) was prepared and treated with trypsin from porcine pancreas (Sigma-Aldrich), papain from papaya latex (Sigma-Aldrich), or a mixture of both enzymes. 2 ml of 4% rabbit erythrocytes in 150 mmol NaCl were mixed with 40 μl of the enzyme stock solution (0.02 mg of enzyme) and incubated for 30 min at 37 \pm 0.5 °C. Three washes were then performed with 150 mmol NaCl.

Lectin thermostability

The protein melting temperature was determined in a 96-well plate by TSA. The lectin was diluted at a concentration of 0.1, 0.2 and 0.5 $\text{mg}\cdot\text{ml}^{-1}$ in PBS and 5X SYPRO Orange® (Bio-Rad) was added in a final volume of 25 μL . The samples were subjected to thermal denaturation in a real time polymerase chain reaction (PCR) machine (Mini Opticon, Bio-Rad) with a temperature gradient from 20 to 100 °C at a heating rate of 1 °C min^{-1} . Protein unfolding was followed by the increase in the fluorescence values given by the SYPRO Orange probe (excitation 450-490 nm and detection 560-580 nm) [41]. Data were evaluated by CFX manager® software (Bio-Rad).

Isoelectric focusing

The pI was determined with a 3.5-10 pH gradient in non-denaturing conditions following Bollag and Edelstein procedure [42].

LBL biotinylation

The lectin was biotinylated [43] with two successive additions of sulfobiotin-X-NHS (Calbiochem) (2:1 w/w, 12 h interval); excess biotin was removed by ultrafiltration using 10 kDa Nanosep filters (Amicon Ultra-15 centrifugal filter devices).

Detection of Tn antigen in cell lines by cellular enzyme-linked immunospecific assay (CELISA)

MCF-7 (human breast adenocarcinoma) and HeLa (cervical cancer) cell-lines (ATCC) were used as positive targets for detecting Tn antigen. The cells were cultured in Dulbecco's modified eagle medium (DMEM), supplemented with 10% bovine fetal serum and antibiotics (50 U $\cdot\text{ml}^{-1}$ penicillin; 50 $\text{g}\cdot\text{ml}^{-1}$ streptomycin and 25 $\text{ng}\cdot\text{ml}^{-1}$ amphotericin) at 37 \pm 0.5 °C in a humid atmosphere and 5% CO_2 . According to the described procedures [24, 44], cells culture (10.000/well) was fixed with 4% paraformaldehyde and then incubated with dilutions serial of biotinylated LBL. Streptavidine-peroxidase (1:500) and H_2O_2 -ABTS were added after suitable washes and finally, absorbance at 415 nm was read on a Bio-Rad ELISA auto reader. Biotinylated VVB₄ 1 mg/ml was used as a control.

Anti SBoL interaction with LBL

The magnitude of interaction between immunoglobulin G (IgG) anti-SBoL [24] and LBL was determined by ELLA. The plates (NUNC, F16 Maxisorp) were sensitized with LBL and SBoL (15 $\mu\text{g}/\text{ml}$) according to the methodology described by Pérez [22]. An IgG stock solution at 100 $\mu\text{g}\cdot\text{ml}^{-1}$ was used for serial dilutions until 100:1 of the final volume in each well. Interactions were detected with a secondary antibody (Anti-IgG) produced in goat and coupled to peroxidase (Sigma-Aldrich) using ABTS as substrate. Absorbance was measured at 415 nm.

RESULTS AND DISCUSSION

Lectin extraction

L. bullata seeds have a black color and hence the protein extracts showed strong pigmentation. Removal of pigments prior protein extraction was, therefore, an essential step in the purification procedure. Different assays were carried out to this end; washing of seeds with a solution of NH_4OH was the most effective. Most remaining pigments were removed using IEC on DEAE-Sephadex in a subsequent purification step. The best extraction results were obtained by including 5 mmol thiourea to the PBS extraction buffer and in the dialysis solutions. It reduced the amount of polyphenols by inhibiting polyphenol oxidases [33] whilst keeping the lectin's activity unaltered (48% in the first extract).

The first extraction gave a very viscous solution presumably resulting from the presence of pectin-like polysaccharides known to be present in nearly all Lamiaceae species and which usually hamper lectin detection [18, 20, 22, 25]. As observed in other studies [18, 20, 25], digestion with Pectinex® permitted to diminish the viscosity and to obtain a significant increase in lectin activity in each extract (table 1). In the first extract, LBL hemagglutination activity got from 48% without Pectinex® treatment to 82% with. All extracts were pooled for the next purification steps. In hemagglutination assay using Tn erythrocytes, the lectin activity was better with crude extracts enzymatically treated (1st, 2nd, 3rd and 4th extracts) than with untreated extracts, showing larger clusters in the first case.

Table 1: ELLA assay activity of crude extracts from *Lepechinia bullata* seeds

Seeds extraction	Lectin activity (%)	
	Untreatment pectinex®	Treatment pectinex®
First extract	48.2 \pm 1.3	82.0 \pm 2.1
Second extract	22.3 \pm 0.6	61.0 \pm 1.4
Third extract	10.7 \pm 0.4	45.0 \pm 0.9
Fourth extract	-	19.3 \pm 0.4
Pool of extracts	34.2 \pm 0.8	45.1 \pm 1.1

Values are means \pm standard deviations (SD) of three determinations.

A crude protein content of 14.4 (\pm 1.1) % in *L. bullata* seeds was obtained, which is higher than for seeds from *S. bogotensis* (13.1%) and lower than for seeds from *S. palifolia* (15.7%) and *S. rubescens* Kunth (19.6%) [25].

In seeds from *Amaranthus caudatus* [45] and *Sesamum indicum* L [46], pertaining to another plants families, has been studied the protein content extracting in water, giving similar results in total protein amount (13.9 % and 14.7 % respectively) than Lamiaceae species.

NaCl addition in extraction solution and pH variation increase the total protein quantity obtaining from seeds [47], however, in almost all cases, these protein isolates were used for protein identification only.

Lectin purification

Precipitation with ammonium sulphate and ethanol 96% was assayed to purify LBL from the pool of extracts but the lectin was

obtained in all fractions (data not shown). Contrary to SBoL [25] and SSL [14] which precipitated at 0-50% and 50-80% ethanol respectively, precipitation could not use as a purification step for

LBL. The extracts were therefore submitted to weak anion exchange chromatography on DEAE-sephadex with elution with 1 M NaCl (fig. 1).

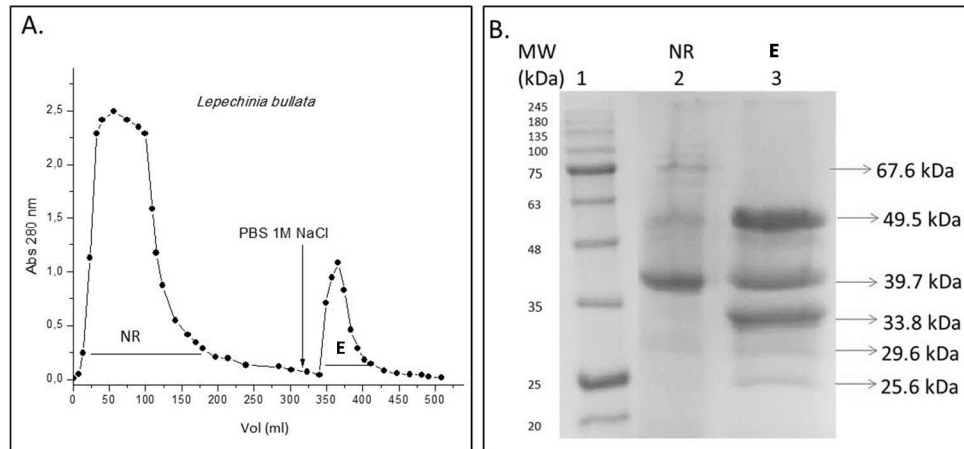


Fig. 1: Chromatographic profile and SDS-PAGE from fractions of *L. bullata* obtained by IEC. NR: Non-retained fraction. E: eluted fraction. MW: molecular weight standard. A. IEC profile at 280 nm. B. 12.5% tris-glycine SDS-PAGE on fractions from IEC. Lane 1. MW; lane 2. Non-retained fraction; lane 3. eluted fraction

The non-retained fraction exhibited hemagglutination on human erythrocytes only with exposed Tn, whilst there was no discrimination for the eluted fraction (table 2). However, both fractions agglutinated enzymatically treated and non-treated rabbit RBCs. The non-retained fraction was thus chosen to continue the purification by affinity

chromatography on aBSM-sepharose. Several coupling methods were assayed and coupling percentage was determined for all affinity supports prepared (table 3). The best purification yield was achieved with 30.2 % of aBSM coupled with cyanogen bromide, indicated that low ligand density was preferable in our case.

Table 2: Hemagglutination activity in LBL fractions from IEC

DEAE fraction	Minimal agglutination concentration ($\mu\text{g}\cdot\mu\text{l}^{-1}$)									
	Human erythrocytes					Rabbit erythrocytes				
	A	B	O	T	Tn	Non-T	T	P	T/P	
Non-retained	-	-	-	-	0.3	0.13	0.13	0.06	0.02	
Eluted	0.09	0.09	0.09	0.02	0.05	0.2	0.2	0.2	0.1	

-Non-agglutination detected, Non-T: Rabbit erythrocytes non-treated enzymatically; T: Trypsin treatment, P: Papain treatment; TP: Trypsin/Papain mixed treatment.

Table 3: Coupling of aBSM to sepharose 4B

Protocol	Coupling (%)	aBSM (mg)/Sephadex 4B (ml)
Cyanogen bromide-activated-Sephadex® 4B	31.0	1.34
ECH-Sephadex® 4B, Carbodiimide method	53.4	0.69
NHS-activated Sephadex™ 4 Fast Flow	57.1	3.12
Sephadex® 4B, reductive amination method	58.2	3.18

The non-retained fraction was devoid of lectin activity, whilst the one eluted with pH 11.4 presented high Tn/T specific hemagglutination activity (fig. 2A, table 4). This fraction presented three protein bands at 28, 48 and 93 kDa on SDS-PAGE in reducing and denaturing conditions (fig. 2B). The lectin activity is conserved with the high score using human and rabbit erythrocytes as observed for MLL and SSL [15, 16].

It was difficult to disaggregate the 93 kDa specie, despite subjection to drastic reducing conditions such as 2 M DTT with heating at 100 °C for 15 min. This behavior was observed previously for SBoL [25] and could be due to the strong alkaline environment obtained during the elution, where the disulfide bonds would be reduced [48] and can be rearranged [49, 50] forming non-native structures [51, 52], contributing to the formation of aggregates [53, 54].

Table 4: Hemagglutination activity in eluted fraction from affinity chromatography

Affinity chromatography	Minimal concentration of protein ($\mu\text{g}\cdot\mu\text{l}^{-1}$) to observe agglutination									
	Human erythrocytes					Rabbit erythrocytes				
	A	B	O	T	Tn	Non-T	T	P	T/P	
Fraction eluted with pH 11.4 (E)	-	-	-	0.07	0.07	0.50	0.25	0.06	0.008	

-Non-agglutination detected, Non-T: Rabbit erythrocytes non-treated enzymatically; T: Trypsin treatment, P: Papain treatment; TP: Trypsin/Papain mixed treatment.

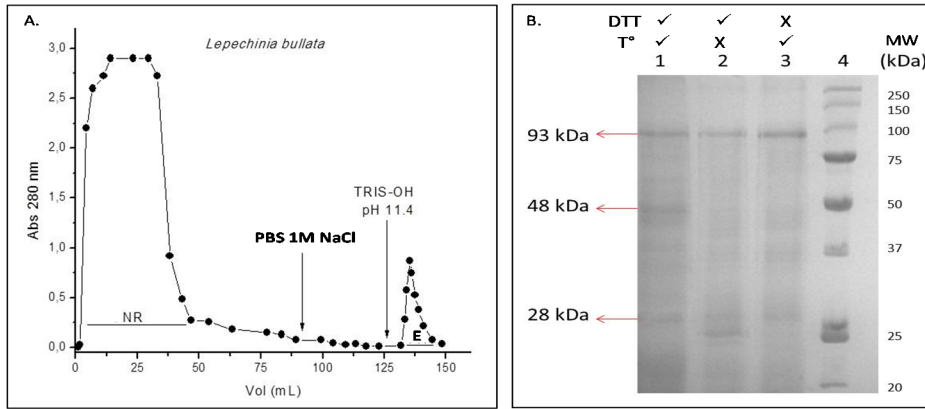


Fig. 2: Chromatographic affinity profile and SDS-PAGE of LBL. NR: Non-retained fraction. E: Eluted fraction. MW: Molecular weight standard. A. Affinity chromatography profile at 280 nm. B. 12.5 % Tris-Glycine SDS-PAGE with eluted LBL at pH 11.4 subjected to different reducing and denaturing conditions. Lane 1. DTT 2 M and heating at 100 °C for 15 min; Lane 2. DTT 2 M without heating; Lane 3. Heating to 100 °C for 15 min, without adding DTT; Lane 4. MW

When purified LBL was subjected to preparative electrophoresis in native conditions, two fractions were obtained which corresponded to monomer and dimer (or high molecular associations), the last form would be the most stable form of LBL (fig. 3A). Both fractions only showed one band at 28 kDa on Tricine-SDS PAGE in reducing and non-reducing conditions (results not shown). Upon reducing conditions in preparative electrophoresis, LBL obtained one fraction only (fig. 3B) in which were observed two bands, one around 31 kDa and another corresponding to a high molecular weight species in Tricine-SDS PAGE under reducing conditions. In otherwise, a single band at 31 kDa without reducing agent but in the presence of SDS, was showed.

In denaturant conditions (fig. 3C) unusual behavior is observed in LBL, where there is a small proportion of a low molecular weight species and a greater proportion of two higher molecular weight associations. This result differs when the preparative

electrophoresis is carried out in the presence of both SDS and DTT, since the monomeric form is not observed, but the high molecular weight species are still present (fig. 3D). In both fractions, a band around 28 kDa was observed on Tricine-SDS PAGE in reducing and non-reducing conditions (results not shown). These results corroborate the absence of interchain disulfide bridges, as well as the presence of reduced intrachain disulfide bonds that cause an apparently higher molecular weight than that determined in native conditions. This behavior may be due to the reduced protein increases the Stokes radius and therefore migrate less in the gel, compared with the folded protein in which disulfide bonds are intact. MLL has three noncovalently linked subunits, under reducing conditions the 42 kDa subunit migrates as a band of 46 kDa [27]. The last fraction lost partially hemagglutinating activity because protein structure is affected by reducing conditions or probably, disulfide intrachain bridges were recombined in a different form regard native form.

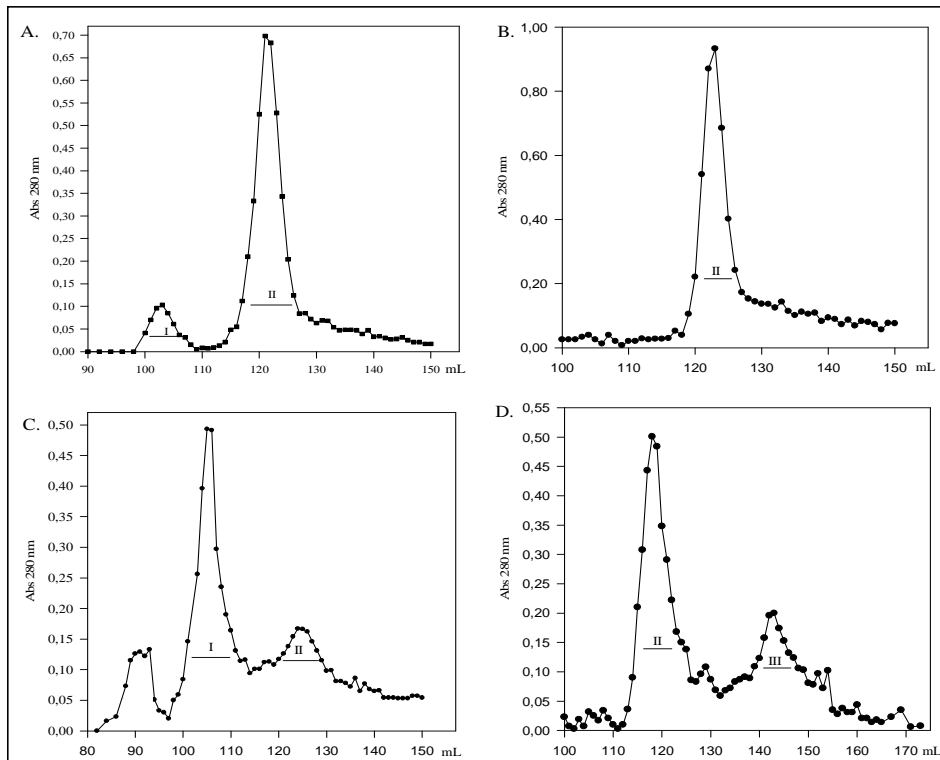


Fig. 3: Preparative SDS-PAGE electrophoresis of LBL. I: Monomeric, II: Dimeric, III: High molecular weight species from LBL. A. Native, B. Reducing (DTT) and heat, C. Denaturing (SDS) and heat and D. Denaturing, reducing (SDS, DTT) and heat conditions in preparative electrophoresis

These results can be explained if consider that the protein has a highly hydrophobic structure with intracatenary disulfide bridges. Studies did on heat effect on β -globulin have shown the formation of aggregates by disulfide bridges exchange and the dimer stabilization by hydrophobic interaction of SDS [53, 54]. Taking into consideration the ensemble of results on the determination of LBL size, we propose that the lectin is associated as a dimer and as a high molecular weight species consisting of non-covalently associated monomers (28 kDa). Lamiaceae lectins corresponds to non-covalently linked subunits, as seen in SBoL [25] and MLL [15, 26, 27], or dimers linked by disulfide bonds like for SSL [14] and *Glechoma hederacea* agglutinin (Gleheda) [17].

Fourth consecutive extractions steps were sufficient for LBL purification since a low lectin activity was detected in the fourth extract treated with Pectinex®. 1339-fold purification was achieved (table 5) which is lower than SBoL (2823-fold) [25] and significantly higher than that for SSL (87-fold) [14]. It corresponds to a lectin amount of 6.2 mg/100 g seeds which is in the range of yield obtained for other lectins as SBoL (18-20 mg/100 g seeds) [25], MLL (15-20 mg/100g seeds) [26], SSL (5 mg/100 g seeds) [14] and Gleheda (10 mg/100 g leaves) [17]. The LBL is insoluble at concentrations higher than 1.0 mg·ml⁻¹ in PBS, while SBoL is soluble at 1.7-2 mg·ml⁻¹[25].

Table 5: Lectin purification from *Lepechinia bullata* seeds

Purification step ¹	Protein (mg/ml) ²	SAA ³	Purification (fold)
1. First extract	2.14±0.1	-	-
2. Second extract	0.81±0.08	-	-
3. Third extract	0.75±0.08	-	-
4. Fourth extract	0.62±0.05	-	-
Pool of extracts	1.11±0.05	3.6	1
5. DEAE (non-retained fraction)	1.36±0.1	5.9	1.6
6. Affinity chromatography	0.43±0.03	4822	1339

¹50 g of seeds extracted with PBS-5 mmol thiourea buffer. ²Determined by the micro-Kjeldahl method. ³SAA is defined as the minimal dilution required for hemagglutination. This assay was done with enzyme-treated A+ erythrocytes to expose Tn antigen [40]. Values are means \pm SD of three determinations.

Previous studies reported that Lamiaceae lectins obtained from *S. sclarea* [14, 16], *Glechoma hederacea* [17], *S. palifolia* [22], *Hyptis mutabilis* [22], *S. bogotensis* [24], *Molucella laevis* [25] and *S. miltiorrhiza* [55] recognize Tn antigen only.

However, in this case, hemagglutination activity was observed with both T and Tn human erythrocytes. LBL recognized rabbit RBCs enzymatically treated and non-treated but was unable to agglutinate human erythrocytes from A, B, O donors and equine or bovine RBCs.

The required minimum lectin concentration was 70 μ g·ml⁻¹ in Tn human erythrocytes and 8 μ g·ml⁻¹ in rabbit RBCs treated with trypsin and papain, which is higher than the one reported for SBoL

(0.17 μ g·ml⁻¹) [25], *S. miltiorrhiza* lectin (SML) (1.99 and 3.91 μ g·ml⁻¹) in mouse and rabbit erythrocytes trypsin-treated respectively, [55] and Gleheda (0.22 μ g·ml⁻¹) [17].

LBL presented a carbohydrate content of 13.4 %, which is in accordance with the 10-15 % found in other Lamiaceae lectins [22] (table 6). Since carbohydrates are usually conserved in plant proteins, the glycan present on LBL should then be similar to Lamiaceae lectins studied to date [22, 25].

Similarly, LBL is also basic since its pI is near to 8.5, which is comparable with the one reported for SBoL (8.8 and 8.6) and SSL (8.8 and 8.0) (table 6).

Table 6: Molecular properties of *L. bullata* and other lamiaceae lectins

Lamiaceae lectins	Recognition	Molecular weight (kDa)		Carbo-hydrates (%)	pI	Disulfide bonds
		Native	Subunits			
<i>Lepechinia bullata</i>	T, Tn, Rabbit RBCs	N. D	93 */** 48*,28*/**	13.4	8.5	Intrachain
<i>Glechoma hederacea</i> [17]	T, Tn	85* 80-93**	26, 28	10	N. D.	Interchain/Intrachain
<i>Hyptis mutabilis</i> [23]	Tn	N. D	55, 51	27.7	7.5 and 6.7	N. D
<i>Molucella laevis</i> [15, 26, 27, 38]	Tn	130	26, 28, 42	10	N. D.	Interchain/Intrachain
<i>Salvia bogotensis</i> [25]	Tn	72	38.7	16	8.8-8.6	Intrachain
<i>Salvia miltiorrhiza</i> [55]	Rabbit and mouse RBCs	N. D	26.3	N. D	4.76	N. D
<i>Salvia palifolia</i> [23]	Tn	N. D	58, 64	23.8	7.4 and 6.8	N. D
<i>Salvia sclarea</i> [14, 16]	Tn	50	35	15	8.8 and 8.0	Interchain

N. D. Not determined. *Reduced protein. **Unreduced protein. + Gel filtration on a superose column. **Sedimentation velocity analysis.

Carbohydrate-binding

Among the assayed sugars and glycoproteins, only GalNAc (75 mmol) and fetuin (25 mmol) completely inhibited the hemagglutination by LBL from Tn human and rabbit erythrocytes respectively (table 7).

Hemagglutination from SBoL, SSL and MLL was inhibited by GalNAc concentrations of 37.5, 0.1 and 0.01 mmol respectively [14, 15, 27]. Gleheda inhibition assays with trypsin-treated rabbit erythrocytes

revealed 50 % inhibition at 25 mmol GalNAc [17]. Fetuin is a highly sialylated glycoprotein, which presents both N and O-glycosylations [56]. In the latter case, the T antigen is sialylated but becomes fully exposed after desialylation with dilute acids [57]. Some ends in fetuin may be desialylated, and thus inhibited the agglutination by LBL of rabbit erythrocytes, indicating the presence and the recognition of Tn antigen.

A comparison of sugar inhibitions in several lectins from Lamiaceae, including LBL, is shown in table 7.

Table 7: Comparison of sugar inhibitions in Lamiaceae lectins

Lamiaceae lectins origin	Inhibitor sugar	Concentration	Model used to assay
<i>L. bullata</i>	GalNAc	75 mmol	Human erythrocytes A+treated enzymatically to expose Tn antigen HeLa and MCF-7 tumoral cell lines
	aMSB	0.2 µg·µl ⁻¹	
	aMSO	0.42 µg·µl ⁻¹	Rabbit erythrocytes treated with trypsin and papain Human erythrocytes A+treated enzymatically to expose Tn antigen
	Fetuin	25 mmol	
<i>S. bogotensis</i> [25]	GalNAc	37.5 mmol	Human erythrocytes A+treated enzymatically to expose Tn antigen
	MSO	0.019 mmol	
	aMSB	0.008 mmol	
<i>S. sclarea</i> [14]	GalNAc	0.1 mmol	Human erythrocytes A+treated enzymatically to expose Tn antigen
	Gal	75 mmol	
	aMSB	0.02 mmol	
	MSB	0.04 mmol	
<i>Moluccella laevis</i> [26]	GalNAc	2.0 µg·ml ⁻¹	Human erythrocytes A ^{MM} and O ^{NN}
	MSO	0.1 µg·ml ⁻¹	
	aMSO	0.003 µg·ml ⁻¹	
<i>Glechoma hederacea</i> [17]	GalNAc	25 mmol	Rabbit erythrocytes treated with trypsin

*Expressed as the concentration of free terminal GalNAc

Effect of temperature and cation

LBL hemagglutination began to diminish above 37±0.5 °C, and was completely abolished at 60 °C. Precipitation of the lectin occurred at temperatures above 80 °C because of thermal denaturation. The lectin's thermal stability which is similar to SSL, appears to be very labile as reported by Piller *et al.* [14]. Other lectins as Gleheda and SBoL retain 50% of their activity at 56 °C [17, 25]. The denaturation temperature could not be determined by TSA perhaps as a result of lectin aggregation or non-binding of the dye in the absence of exposed hydrophobic residues, corroborating bands obtained in SDS-PAGE. All tested concentrations led to a similar result.

LBL was active in PBS or 1% NaCl at RT without requiring metals addition, but in presence of EDTA lectin's activity decreased by 50%, indicating it depends on the presence of divalent ions. Other lectins such as some of the Legume present the same behaviour [30, 58]. These proteins are composed of two or four identical subunits with a molecular weight between 25 and 30 kDa, each of which contains a binding site for the Ca²⁺, Mn²⁺ and/or Mg²⁺ metals in nearby to "carbohydrate bind site" [59]. A typical example are lectins from Viceae and Diocleae tribes, specially Concanavalin A [60]. Maybe in

the structure of LBL, the interaction between ions and the protein is weak, or the ions are exposed and are therefore easily removed by the action of chelating agents. The lectin's activity depends on Ca²⁺ and Mn²⁺ binding to the native protein, as well as the presence of intact disulphide bridges as observed for SSL and SBoL [14, 25].

The amino acid composition (table 8) showed a high content of glycine, lysine, histidine and hydroxy amino acids. The protein contains methionine, unusual residue among legume lectins and some Lamiaceae. The five cysteine residues apparently form intrachain disulphide bonds, given that reduced as well as non-reduced samples showed almost the same bands by SDS-PAGE. Something similar occurs in Gleheda, where there are three cysteines, two of which forming one intrachain disulfide bridge [17]. According to Alperin *et al.* [26], no disulfide bond is present in 26 kDa subunit from MLL, whereas an interchain disulfide bond links the two subunits for SSL and Gleheda [14, 16, 17].

Considering the pI of LBL of 8.5, a great proportion of Asx and Glx should correspond to asparagine and glutamine respectively, and there is also a great content of lysine and histidine to explain its basic character.

Table 8: Amino acid composition of lectins from *L. bullata* and other lamiaceae species

Amino acid (AA)	LBL ¹		SBoL ⁵		SSL ⁶		SML ⁷		Gleheda ⁸	
	mg AA/100 g protein	Calculated residues	Nearest integer ⁴	Residues in polipeptide chain	Calculated residues	Residues in polipeptide chain	Residues in polipeptide chain	Residues in polipeptide chain		
Ala (A)	2.44	8.3	8	36	75.9	21	16			
Cys (C)	2.07 ²	4.9	5	4	1.2	0	3			
Phe (F)	9.08	14.9	15	4	36.6	15	11			
Gly (G)	8.63	36.7	37	92	126.8	22	20			
His (H)	10.79	19.1	19	4	9.4	5	9			
Ile (I)	4.48	9.6	10	6	20.6	13	16			
Lys (K)	10.61	20.1	20	21	7.8	4	14			
Leu (L)	7.12	15.2	15	10	27	13	15			
Met (M)	1.05 ²	1.7	2	0	1.3	2	4			
Pro (P)	2.88	7.2	7	13	17.7	10	11			
Arg (R)	4.30	6.7	7	3	13.2	8	6			
Ser (S)	4.39	12.2	12	60	73.2	22	20			
Thr (T)	4.37	10.4	10	13	66.2	26	19			
Val (V)	4.85	11.9	12	10	43.9	25	21			
Trp ³ (W)	7.78	10.1	10	9	N. D.	3	5			
Tyr (Y)	5.70	8.4	8	5	12.4	8	4			
Asx (D+N)	6.85	14.4	14	15	58.6	27	32			
Asp (D)	N. D.	N. D.	N. D.	N. D.	N. D.	14	19			
Asn (N)	N. D.	N. D.	N. D.	N. D.	N. D.	13	13			
Glx (E+Q)	5.54	10.4	10	42	31.2	22	10			
Glu (E)	N. D.	N. D.	N. D.	N. D.	N. D.	7	5			
Gln (Q)	N. D.	N. D.	N. D.	N. D.	N. D.	15	5			

N. D. Non determined. ¹Calculations based on a Mr = 28 000 g with 13,4 % carbohydrate. ²Determined as MetSO₂ and CySO₃. ³Determined spectrophotometrically. ⁴Residues per polypeptide chain. ⁵Taken from Vega and Pérez [25]. ⁶Calculated from Medeiros *et al.* [16]. Mr = 60 000 Da. ⁷Based in Wenping *et al.* [55]. ⁸Based in Wang *et al.* [17].

LBL interaction with HeLa and MCF-7 Cells

Considering LBL's ability to specifically recognize the Tn/T antigen, specific recognition of Tn determinant was corroborated by CELISA, in which biotinylated LBL strongly bound to HeLa Cells (fig. 4A). VVB₄ was used as a control due to its well-established Tn specificity [28]. A strong specific binding towards the two cell lines was revealed in the same extend than VVB₄ just using 2.5 µg of each lectin. 50% of interaction was inhibited using 492 µg of GalNAc, 0.42 µg of OSM (13.8-15% GalNAc content) and 0.2 µg of aBSM (18% GalNAc content). SBoL binding to MCF-7 (breast cancer), HeLa (cervix cancer) and BHK (kidney fibroblasts) cell lines in periodontal tissue and mice skin fibroblast primary cultures, has been described previously [24]. Osinaga *et al.* [61] detected Tn antigen by using a

monoclonal antibody (83D4) and the inhibition assays were performed by using lectins as inhibitors, the best inhibitor was VVB₄. Therefore, lectins are valuable tools for the detection of antigen Tn.

Anti-SBoL interaction with LBL

5 µg of Anti-SBoL IgG were necessary for LBL recognition (fig. 4B). These results indicate that the two proteins could have similarity structural and sequence since the antibody almost identically recognizes them. Similar results were obtained using anti-SBoL IgY in order to detect interaction with *Salvia hispanica*, *L. bullata* and Leguminosae lectins; a somewhat lesser interaction was observed with LBL showing this lectin lesser structural homology with SBoL [62].

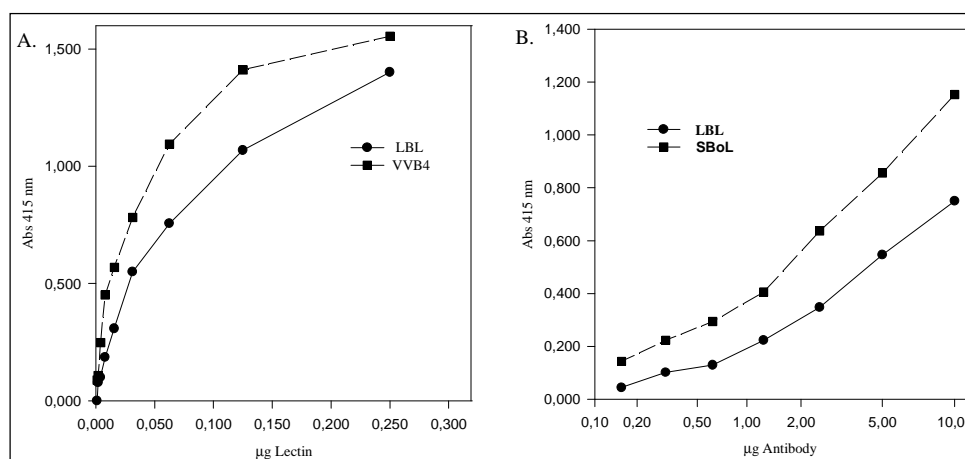


Fig. 4: Interaction of LBL with Cell line HeLa and anti-SBoL. A. Tn detection in HeLa Cell line. B. Anti-SBoL antibody interaction with LBL by ELLA

CONCLUSION

The lectin's specificity and high affinity for the T and Tn antigens, commonly found in tumor cells, makes this protein a useful tool for immuno-histochemical and cellular studies. Our future aim is to carry out structural studies on Lamiaceae lectins to know about interaction lectin-T/Tn antigens and how this molecule could be used like a tool in clinical assays. To date, this field remains unexplored.

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AUTHORS CONTRIBUTION

Author name	Research conception/ design	Data acquisition	Data analysis/ Interpretation	Manuscript preparation	Final approval
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José Rojas-Caravallo	X	X	X		
Edilma Sanabria	X	X	X		
Edgar Reyes-Montaño			X	X	
José Luis Fernández-Alonso			X	X	X
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CONFLICT OF INTERESTS

Declared none

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