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Isolation and characterisation of a *Salvia bogotensis* seed lectin specific for the Tn antigen

Nohora Vega, Gerardo Pérez *

Biochemistry Laboratory, Chemistry Department, Universidad Nacional de Colombia, Bogotá, Colombia

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Abstract

A lectin was isolated and characterised from *Salvia bogotensis* seeds. Removal of the abundant pigments and polysaccharides, which are present in seeds, was an essential step in its purification. Several procedures were assayed and the best suited, including Pectinex treatment, DEAE-cellulose and affinity chromatography, led to a protein being obtained amounting to 18–20 mg/100 g seeds having high specific agglutination activity (SAA). The lectin specifically agglutinated human Tn erythrocytes and was inhibited by 37 mM GalNAc, 0.019 mM ovine submaxillary mucin (OSM) or 0.008 mM asialo bovine submaxillary mucin (aBSM). Enzyme-linked lectinosorbent assay (ELLSA) revealed strong binding to aOSM and aBSM, corroborating Tn specificity, whereas no binding to fetuin or asialo fetuin was observed. The lectin's monomer MW (38,702 Da), amino acid composition, p*I*, carbohydrate content, deglycosylated form MW, thermal stability and Ca^{2+} and Mn^{2+} requirements were determined. Evidence of the existence of two glycoforms was obtained. The lectin's specificity and high affinity for the Tn antigen, commonly found in tumour cells, makes this protein a useful tool for immunohistochemical and cellular studies.

Keywords: *Salvia bogotensis*; Lamiaceae; Lectin; Characterisation; Isolation; Tn antigen

1. Introduction

In the course of their research into lectins, Bird's group (Bird and Wingham, 1974; Bird and Wingham, 1976, 1977) found lectins able to specifically recognise the Tn antigen (GalNAc-O-Ser/Thr) in several Old World species of Salvia (Lamiaceae); this antigen is responsible for the erythrocyte polyaggutinability shown by some individuals and has been identified as being a tumour cell marker (Springer, 1984; Lisowska, 1995). The latter is useful in diagnosis when following-up the evolution of several types of cancer. The Tn epitope has also been detected on human immunodeficiency virus gp160 and gp120 proteins (Hansen et al., 1991).

Detailed studies, using Lamiaceae lectins, have been carried out on a few species from the Northern hemisphere's temperate zone. The lectin from *Salvia sclarea* seeds (SSL) was the first to be isolated and partially characterised (Piller et al., 1986). This established its specific binding to

Abbreviations: ABTS, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); aBSM, asialo bovine submaxillary mucin; BCA, bicinchoninic acid; BSA, bovine serum albumin; BSM, mucin; ConA, Concanavalin A; DAB, diaminobenzamidine; DDCA, diethyldithiocarbamic acid; DTT, dithiothreitol; EGTA, ethylene glycol-*O*,-*O*′-bis (2-amino-ethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ELLSA, enzyme-linked lectinosorbent assay; EME, enzymatically modified erythrocytes; ERL, *Erythrina rubrinervia* lectin; SBoL, *Salvia bogotensis* lectin; SSL, *Salvia sclarea* lectin; MeCN, acetonitrile; MLL, *Moluccella laevis* lectin; GLL, *Galactia lindenii* lectin; DLL-II, *Dioclea lehmanni* lectin II; OSM, ovine submaxillary mucin; aOSM, asialo ovine submaxillary mucin; PVPP, polyvinylpolypyrrolidone; RBC, red blood cell; RT, room temperature; SAA, specific agglutination activity; SDS, sodium dodecyl sulphate; VVB4, *Vicia villosa* isolectin B4; TBS, Tris buffer saline; TFA, trifluoroacetic acid; Gleheda, *Glechoma hederacea* lectin.

* Corresponding author. Tel.: +57 1 3165000x14465/14470; fax: +57 1 3165220.
E-mail address: jrperezg@unal.edu.co (G. Pérez).

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both native Tn red blood cells (RBCs) and enzyme-treated RBCs, as well as the inhibitory potential to a variety of carbohydrates, Synsorb-coupled synthetic glycopeptides, BSM, and aBSM. Several molecular features of SSL have been described, such as MW and N-linked oligosaccharide structures (Medeiros et al., 2000); competition binding studies with soluble synthetic glycopeptides have also helped to define the density requirements of Tn structures.

A lectin from *Moluccella laevis* (MLL) has been isolated (Lis et al., 1988). Besides recognising A^{MM} and O^{NN} erythrocytes, the lectin binds strongly to Tn-bearing glycoproteins (Duk et al., 1992), Tn-bearing lymphocytes (Thurnher et al., 1993) and glycosphingolipids (Teneberg et al., 1994). Analysis of the lectin's structural features revealed a unique subunit composition (Alperin et al., 1992), making it an unusual lectin. Wang et al. (2003a) have recently found a lectin (Gleheda) in *Glecoma hederacea* leaves which readily interacts with *O*-glycans linked to asialo mucin or asialo fetuin in which Gal/GalNAc are terminally exposed. The authors used sequence and molecular modelling studies to demonstrate that Gleheda is structurally and evolutionarily related to legume lectins. A potentially interesting development arises from the insecticidal properties presented by the lectin (Wang et al., 2003b).

Botanical studies have revealed the presence of around 190 Lamiaceae species in Colombia, Salvia being the most diverse genus as it has 75 species (Wood and Harley, 1989; Fernández-Alonso, 2003). We have recently carried out an extensive survey of six genera and 40 taxa as no data was available concerning the presence of lectins in Colombian species of Lamiaceae (Fernández-Alonso et al., 2003). This study revealed both the presence of lectins able to recognise the Tn antigen in more than 80% of the studied species and remarkable differences in lectin activity within a given genus (i.e. eight out of 19 *Salvia* species had more than 80% activity levels). Considering the potential applications of anti-Tn lectins, we chose the *Salvia bogotensis* (Benth.) species for this study, taking its endemic character into account (wide distribution throughout the eastern Colombian Cordillera), the availability of substantial amounts of seeds and its high lectin activity (98%). This work describes isolating and characterising *S. bogotensis* lectin (SBoL) as a first step in studying its interaction with Tn-bearing cells.

2. Results and discussion

2.1. Lectin extraction

Removing troublesome abundant pigments which usually appear in Lamiaceae seed protein extracts was an essential purification step when obtaining lectin. The problems pertained to reduced protein solubility, inadequate assessment of elution profiles if followed by absorption at 280 nm and inaccurate determination of $A^{280}_{1\%}$ values which could be used for calculating lectin content in seeds.

Treatment with 0.1 M ascorbic acid, 2% polyvinylpolypyrrolidone (PVPP) or 0.5% diethyldithiocarbamic acid (DDCA) appreciably reduced the lectin's activity (data not shown) with no significant reduction of pigment in the extract. This loss of activity, particularly with PVPP, has been observed in our laboratory with other proteins (*Salvia palifolia* or *Hyptis mutabilis* lectins); it is likely that this was due to protein adsorption on PVPP. The total loss of activity when including dithiotreitol (DTT) indicated the presence of disulphide bridges in the protein.

The best results were obtained by including 5 mM thiourea in PBS pH 7.2 and in dialysis solutions, which reduced the amount of polyphenols by inhibiting polyphenol oxidases (Van Driessche et al., 1983) whilst keeping the lectin's activity unaltered (79%). Most remaining pigments were effectively removed by DEAE-cellulose and DEAE-Sephadex during subsequent purification steps. This approach has proved very effective in our hands when working with highly pigmented extracts from several Lamiaceae species. It is likely that improved yields and higher specific agglutination activities than those obtained with previously described Lamiaceae seed lectins (Piller et al., 1986; Alperin et al., 1992) have been due to the isolation procedure described in this work.

There was 2.09% nitrogen content in seeds, amounting to 13.1% crude protein which is lower than that of most legume seeds; with the exception of *S. palifolia* (15.7%) and *S. rubescens* (19.6%) (Filgueira and Aldana, personal communication), no data is currently available for other *Salvia* seeds. Non-protein nitrogen accounted for 0.11%; net protein content in seeds was thus 12.3%.

2.2. Lectin purification

Precipitation assays using PBS extracts showed that the lectin precipitated at 50% ethanol presenting 70–80% activity as determined by ELLSA. Higher ethanol concentrations did not precipitate the lectin further as opposed to SSL behaviour which precipitated at 80% ethanol (Piller et al., 1986). Dissolving the resulting precipitate led to a very viscous solution being formed, due to pectin-like polysaccharides which are present in nearly all *Salvia* species and which usually hamper detecting lectin (Fernández-Alonso et al., 2003); viscosity diminished after digestion with Pectinex and chromatography over DEAE-Sephadex (at an improved flow) yielded a non-retained peak in which lectin activity was readily detected (52%), even at low protein concentrations.

Affinity chromatography on aBSM-Sepharose 4B of the non-retained DEAE-Sephadex peak yielded two fractions (Fig. 1a); the first (I) was devoid of lectin activity (6.7%, 1.9 mg protein/ml) and the second (II), eluted by pH 11.4, presented 71–78% activity (0.4–0.5 mg protein/ml) after dialysis, as well as high Tn-specific agglutination activity (Table 1). The minor peak eluting before fraction II was devoid of lectin activity and was therefore discarded.

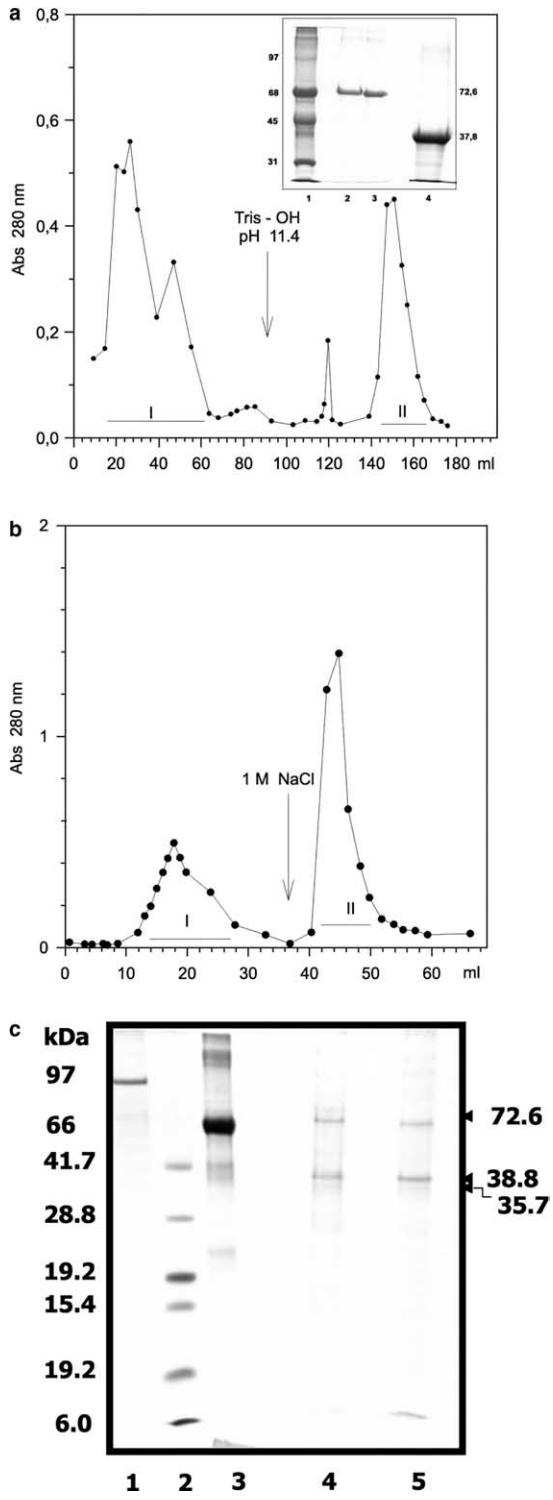

Fig. 1. *Salvia bogotensis* lectin chromatography on aBSM (a), DEAE-Sepharose (b) and Tricine-PAGE (c) of purified lectin. (a) Lectin-active fraction I from DEAE-Sephadex column was applied to an aBSM-Sepharose 4B column yielding lectin-active fraction II. Inset: SDS–PAGE of fraction II. MW standards (lane 1); non-reduced (15 μg, lane 2); mild reducing conditions (40 mM DDT, 3 min) (15 μg, lane 3); strong reducing conditions (2 M DTT, 30 min) (30 μg, lane 4). (b) Fraction II was applied to a DEAE-Sepharose 4B column yielding lectin-active fraction I. (c) Tricine-PAGE of DEAE-Sepharose 4B fraction I. MW standards (lanes 1–3); fraction I under mild reducing conditions (lane 4), fraction I under non-reducing conditions (lane 5).

Subsequent chromatography on DEAE-Sepharose (Fig. 1b) allowed a protein having similar activity (ELLSA) and higher specific agglutination activity to be recovered from the non-retained fraction (I) (Table 1) which was devoid (after concentration) of contaminating pigments. This fraction had two major bands at 72.6 kDa (33% total protein) and 38.8 kDa (54.2%) and a minor band (12.6%) at 35.7 kDa by Tricine-PAGE (Fig. 1c) in reducing/non-reducing conditions; the two close bands were probably lectin glycoforms. Table 1 summarises the results obtained during the purification steps. Three consecutive extractions were sufficient as very low lectin activity was detected in the fourth extract. The extracted protein (albumins and globulins) amounted to 22.5% of total protein in seeds. The solution resulting from treatment with DEAE-cellulose was faintly coloured with the protein remaining dissolved (after concentration) at 3–4 mg/ml; 2823-fold purification was achieved (Table 1), being significantly higher than that for *S. sclarea* lectin (87-fold) (Piller et al., 1986). Complete removal of pigments after further chromatography on DEAE-Sepharose led to protein solutions being obtained in subsequent assays in the 1.7–2 mg/ml range (after concentration) without potentially interfering agents. The data presented in Table 1 was taken into account when calculating a lectin amount of 18–20 mg/100 g seeds, this being similar to MLL (15–20 mg/100 g seeds; Alperin et al., 1992) and higher than SSL (5 mg/100 g seeds; Piller et al., 1986) or Gleheda (10 mg/kg leaves; Wang et al., 2003a).

2.3. Agglutination of human and animal erythrocytes

The lectin was unable to agglutinate human RBCs from A, A_1, B and O donors or T erythrocytes obtained after enzymatic treatment; only Tn erythrocytes became agglutinated. Minimum required lectin concentration was 0.17 μg/ml, thus being more potent than Gleheda lectin (Wang et al., 2003a); no difference was observed among Rh+ and Rh− cells. No agglutination was detected with rabbit, cow, horse, or dog RBCs (even at 1.48 mg lectin/ml), apart from *Lepechinia bullata* lectin which presented identical behaviour (Rojas, personal communication). In this respect, no data is currently available for other Lamiaceae lectins.

2.4. Carbohydrate and glycoprotein binding

Amongst the assayed sugars (ca. 35) only GalNAc (37.5 mM) completely inhibited Tn erythrocyte agglutination by SBoL; specific interaction with Tn determinant was advanced in the affinity chromatography experiments described above with aBSM-Sepharose. Erythroagglutination by SSL and MLL was inhibited by lower GalNAc concentrations (0.1 mM and 0.03 mM, respectively) and by P-NO_2 Phenyl-α/β D-GalNAc (0.12/0.06 mM and 1.75/0.87 mM, respectively) (Piller et al., 1986; Lis et al., 1988; Lis and Sharon, 1994) whereas Gleheda inhibition assays with trypsin-treated human RBCs revealed 50% inhibition at 25 mM GalNAc (Wang et al., 2003a).

Table 1
Salvia bogotensis seed lectin purification

Purification step [a]	Protein (mg/ml)	Vol (ml)	Total protein (mg)	Specific agglutination activity[b]	Purification (fold)
1. First extract	2.56	530	1356.8	–	–
2. Second extract	0.83	666	552.8	–	–
3. Third extract	0.44	578	254.3	–	–
Pool of extracts [c]	0.96	1589	1525.4	480.0	1
4. 50% ethanol	1.48	446	660	17.3	27.7
5. DEAE-peak I [d]	0.044	116	5.1	2.8	171.4
6. Affinity chromatography [e]	0.051	20	1.02	0.24	2000
7. DEAE-Sepharose chromatography [f]	0.09	16	1.44	0.17	2823.5

[a] 76.5 g of seeds extracted with PBS-5 mM thiourea buffer.
[b] The specific agglutination activity is defined as the minimal protein concentration (μg/ml) required for agglutination. This assay was done with enzyme-treated A+ erythrocytes (Hirohashi et al., 1985).
[c] After pigment removal by DEAE-cellulose.
[d] 37 mg of protein was applied to the DEAE-Sephadex column.
[e] 5.6 mg of protein was applied to the aBSM-Sepharose 4B column.
[f] 1.8 mg of protein was applied to the DEAE-Sepharose 4B column.

Erythroagglutination was also inhibited by ovine submaxillary mucin (OSM) (13.8–15% GalNAc content) and aBSM (18% GalNAc content) at glycoprotein concentrations equivalent to 19 and 8 μM GalNAc, respectively; similar aBSM concentrations inhibited erythroagglutination by SSL (Piller et al., 1986). Specific recognition of the Tn determinant was corroborated by ELLSA assays in which biotinylated SBoL bound strongly to aOSM (Fig. 2). WB4 was used as control due to its well-established Tn specificity (Tollefsen and Kornfeld, 1983). As expected, binding to OSM was significantly lower due to the low content of exposed Tn determinants in natural mucins.

As expected, no lectin binding to Fetuin-Agarose or asialo Fetuin-Agarose was observed by affinity chromatography since Tn antigen is not exposed in them; weak interaction was detected with asialo agalacto Fetuin-Agarose as two low-absorbing retained fractions could be recovered by elution at pHs 2.5 and 11.4 (results not shown). Although these fractions showed some activity, they were heterogeneous by SDS–PAGE and their study was not pursued further. The weak binding to asialo agalactoFetuin was due to a lower antigen density than in aBSM or aOSM (Wu et al., 2003).

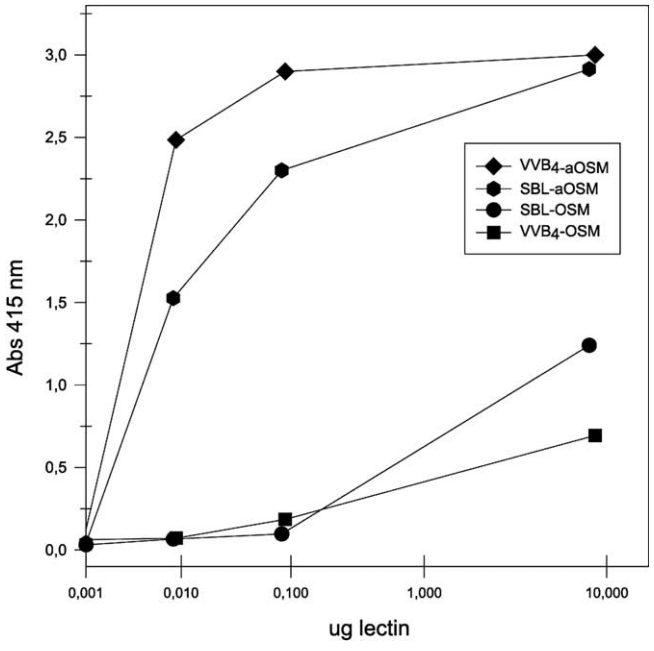

Fig. 2. *Salvia bogotensis* lectin and *Vicia villosa* B4 isolectin binding to OSM and aOSM. ELLSA assays were carried out as described in Section 3.

2.5. Effect of pH, temperature and cation

Maximum pH stability of SBoL was reached at pH 7–8 (SAA 0.44 μg/ml) and close to complete loss of activity was only observed (results not shown) at low (pH 2.0) or high (pH 12.5) pH values (SAA 14-28 μg/ml). Activity was not recovered when the sample exposed to pH 12.5 was brought back to pH 7.0.

Assessing the effect of temperature showed that protein activity began to diminish above 40 °C, becoming completely eliminated at 92 °C. The lectin's thermal stability is remarkable as it retains 50% of its activity at 56 °C, this being similar to Gleheda (Wang et al., 2003a); on the contrary, SSL appears to be very labile as reported by Piller et al. (1986). It should be stressed that the protein remained active after two years' storage in 50% glycerol at −20 °C.

SBoL was fully active in PBS or 1% NaCl without requiring the addition of metals. Specific agglutination activity diminished from 0.44 μg/ml to 28.5 μg/ml following demetallisation. Recovery of activity was minimal after re-equilibration with 0.1 M $CaCl_2$ or 0.1 M $MnCl_2$ since final SAA were 14.25 μg/ml and 3.56 μg/ml, respectively. The lectin's activity therefore depended upon Ca^{2+} and Mn^{2+} being bound to native protein as well as on intact disulfide bridges, being similar to SSL in this respect (Piller et al., 1986).

2.6. Molecular properties

Table 2 summarises the molecular properties of SBoL as well as those of hitherto characterised Lamiaceae lectins.

SDS-PAGE of biotinylated SBoL showed a 72.6 and 37.5 kDa band following glycoprotein staining using DAB in the detection system (Amersham, 2001); the protein had 16% carbohydrate. Sugar content is 10%–15% in Lamiaceae lectins described to date (Piller et al., 1986; Lis and Sharon, 1994; Wang et al., 2003a). Tricine-PAGE analysis of enzymatically deglycosylated SBoL (10 μg) revealed the presence of a major 40.1 kDa band (40.7%), as well as 72.6 kDa (22.6%) and 35.7 kDa (36.7%) bands (results not shown). This indicated that PNGase F deglycosylation was partial. The 4.4 kDa difference in molecular weight between glycosylated and deglycosylated monomer agreed with 16% carbohydrate content. SBoL carbohydrate content, deglycosylated protein MW, lectin binding by ConA (which binds glycans with a "mannose core") and the hypothesis that SBoL glycan is similar to that of Gleheda (in which 6–8 monosaccharide units are present with a "mannose core"; Wang et al., 2003a) lead us to proposing a glycan-like structure having a 3–4 glycan/polypeptide chain and the likely existence of two glycoforms detectable by Tricine-PAGE as closely migrating bands, the 38.8 kDa band being predominant. Two to three oligosaccharide chains are present in SSL (Medeiros et al., 2000) and 1–2 *N*-glycan per polypeptide chain have been proposed in Gleheda (Wang et al., 2003a).

SDS–PAGE analysis of aBSM-Sepharose 4B fraction II (Fig. 1a, inset) consistently revealed a 70–72 kDa band as being a major constituent (lane 2); this band was resistant to ordinary reduction conditions (40 mM DTT, 3 min) (lane 3), drastic conditions (2 M DTT, 30 min) (lane 4) being needed to halve its size. The evidence suggested that S–S interchange, likely due to the basic pH employed in the purification, had taken place to some extent. Occurrence of this interchange at basic pH has been well documented (Spackman et al., 1960) and, in our case, probably led to the 72.6 kDa band which presented S–S bonds fairly resistant to usual reduction conditions. It should be noted that Lis et al. (1988) observed MLL aggregation in PBS and that there is MS evidence of a similar situation for SSL as a 60 kDa protein fraction is still present after reduction (Medeiros et al., 2000). Estimating MW by gel filtration was not feasible as abnormally high elution volumes were observed with Spherogel TSK 3000 SW, Sephacryl S-200, Biogel P150 and Superose 12 which was most likely due to protein interaction with the support.

Reduction and carboxymethylation of the protein yielded a precipitate which was shown by Western blot as a band at 72.6 kDa (when using anti-SBoL antibody); the supernatant showed a 38.8 kDa band and 35.7 kDa band (results not shown). This result corroborates the formation of a dimer that resists reducing conditions.

ES-MS analysis showed a 38,702 ± 22 Da mass protein by tandem spectroscopy; this value agrees very well with that of the main band observed by Tricine-PAGE for the purified protein (Fig. 1c) or that obtained after drastic reduction (Fig. 1a, inset lane 4) corresponding to the lectin monomer. MALDI-TOF MS analysis of SSL yielded a broad peak having 60–61 kDa mass (Medeiros et al., 2000) considered to be the protein's dimer form since it had a 29.5–30 kDa mass after reduction and carboxymethylation. Taking the ensemble of results pertaining to determining size into consideration, we propose that the lectin is a dimer consisting of non-covalently associated monomers (38 kDa). In view of the existing evidence, the subunit arrangement in Lamiaceae lectins either corresponds to non-covalently linked subunits, as in SBoL and MLL, or dimers linked by S–S bonds, such as SSL and Gleheda.

2.7. Amino acid analysis

Table 3 shows SBoL amino acid composition. Besides the high amounts of Ser, Glx, Gly, Ala and Lys, it is noteworthy that four 1/2 Cys were present. Taking into account that no free CySH was detected and that the results obtained by Tricine-PAGE showed no change in M_r before and after mild reduction (Fig. 1c), the four 1/2 Cys most likely form two intra-catenary disulfide bridges. According to Alperin et al. (1992), no S–S link is present in the MLL 26 kDa subunit, whereas an interchain S–S bond links the two subunits for SSL and Gleheda (Medeiros et al., 2000; Wang et al., 2003a). Considering that the protein p*I* is 8.6–8.8, a considerable proportion of Glx and Asx should

Table 2
Molecular properties of *Salvia bogotensis* and other Lamiaceae lectins

	S. bogotensis	*S. sclarea*[a,b]	*G. hederacea*[c]	*M. laevis*[d]
M_r subunits	38.8; 35.7 [e]kDa	32 [b]kDa	26–28 kDa	26 kDa
M_r protein	38,702 Da	60–61 kDa[b]	80–93 kDa	130 kDa
Bands in SDS–PAGE	72.6; 38.8;	50; 35 kDa[a]	26 kDa (66%)	67; 42
	35.7 kDa	72; 32 kDa[b]	28 kDa (34%)	26 kDa
Neutral sugars	16%	15%[a]	10%	10%
p*I*	8.6–8.8	8.8, 8.0[a,e]; 5.5[b]	6.11	ND

[a] Piller et al. (1986).
[b] Medeiros et al. (2000).
[c] Wang et al. (2003a).
[d] Alperin et al. (1992).
[e] Minor band.

Table 3
Salvia bogotensis lectin amino acid composition

Amino acid	*Salvia bogotensis*			*S. sclarea*[a]	*G. hederacea*[b]
	AA/100 g protein	Calculated residues/mol	Nearest integer[c]	Calculated residues/mol	Residues/mol
Asx	5.42	15.3	15	58.6	31
Thr	3.98	12.8	13	66.2	19
Ser	16.03	59.8	60	73.2	20
Glx	16.55	41.7	42	31.2	10
Gly	16.06	91.5	92	126.8	20
Ala	7.94	36.3	36	75.9	16
Val	2.95	9.7	10	43.9	20
Met	0.2[d]	0	0	1.3	4
Cys	1.22[d]	3.9	4	1.2	3
Ile	2.22	6.4	6	20.6	16
Leu	3.46	9.9	10	27	15
Tyr	2.30	4.6	5	12.4	4
Phe	1.81	4.0	4	36.6	12
His	1.55	3.7	4	9.4	9
Lys	8.28	21.0	21	7.8	14
Arg	1.29	2.7	3	13.2	6
Pro	3.7	12.7	13	17.7	11
Trp[e]	5.1	8.9	9	ND	5

Calculations are based on a $M_r = 38{,}702$ with 16% carbohydrate.
[a] Calculated from Medeiros et al. (2000). $M_r = 60{,}000$.
[b] Wang et al. (2003a).
[c] Residues/polypeptide chain.
[d] Determined as $MetSO_2$ and $CySO_3$.
[e] Determined spectrophotometrically.

correspond to AsN and GlN. Attempts to determine the N-terminal sequence were unsuccessful because the N-terminus was apparently blocked. Apart from Gleheda, no N-terminal sequence has yet been determined for any Lamiaceae lectin.

The high amount of recovered protein and the devised, simple method for isolating the lectin showed that *S. bogotensis* seeds represent an excellent source for obtaining a Tn-specific lectin which can be used as a tool for immunohistochemical and cellular studies concerning the presence of this antigen in tumours.

3. Experimental

3.1. Chemicals and plant material

S. bogotensis (accession number: COL 422764) seeds were collected from the Mondoñedo area, near Bogotá. Fresh human blood was obtained from the U. Nacional's Clinical Laboratory. Animal erythrocytes were supplied by the Veterinary Faculty's Haematology Laboratory. Pharmacia and BioRad equipment were used for chromatography. ConA-Sepharose, Fetuin-Agarose and proteins used as standards were all from Sigma. Bovine submaxillary mucin and ovine submaxillary mucin were from Fluka. Sugars were commercial products having the highest available purity. The rest of the reagents were analytical grade.

3.2. General methods

Mature *S. bogotensis* seeds were ground to a fine powder; the resulting flour was subjected to preliminary analysis (humidity, nitrogen, ash, lipid and crude fibre content). Non-protein nitrogen was determined according to Pérez et al. (1990).

Carbohydrate inhibition and demetallisation assays and neutral sugar, amino acid composition and extinction coefficient determination were performed by those methods cited by Pérez (1984). Final carbohydrate concentration was 100 mM in most cases, except for α- and β-*p*-nitrophenyl glucose, galactose or mannose derivatives which were 12.5 mM. The lectin was dialysed against PBS pH 7.0 in the demetallisation assay (after dialysis against 50 mM EDTA); erythroagglutinating activity was tested in the presence and absence of 5–100 mM Ca^{2+} and Mn^{2+} (chloride ions). Specific agglutination activity was determined as being the minimal protein concentration (μg/ml) required for agglutination. Protein was determined by microKjeldahl, a modified Bradford method (Stoscheck, 1990) or by bicinchoninic acid (BCA) assay. Erythroagglutination assays were done on human and animal RBCs as described (Pérez, 1984) and on T/Tn-exposed RBCs. A+ human RBCs were enzymatically treated to expose T or Tn determinants (Hirohashi et al., 1985).

3.3. Lectin extraction

Several procedures were assayed for removing pigments or diminishing their formation due to the seeds' high polyphenol content. The final procedure was as follows. In small scale experiments, seeds (1 g) were left to soak in 20 mM sodium phosphate buffer – 150 mM NaCl (PBS) – 5 mM thiourea, pH 7.0–7.2 (Van Driessche et al., 1983) for 2–3 h, at 4 °C; they were then macerated and extracted with continuous stirring at 4 °C, for 16 h. The extract (having a viscous appearance) was centrifuged (38,000*g*, 1 h, 4 °C). Supernatant haemagglutination activity was determined and the supernatant was used immediately or treated with Pectinex.

As a result of the previous assays, extractions were then done on a larger scale (50–70 g) with PBS-5 mM thiourea buffer; three successive extractions were done in the conditions described. An aliquot (10–20 ml) of each extract was taken for nitrogen determination by micro-Kjeldahl. The extract pool was extensively dialysed against PBS-5 mM thiourea buffer and tested for haemagglutination and lectin activity (ELLSA).

3.4. Pectinex treatment

Extract pH was adjusted to pH 4.7 with concentrated AcOH; Pectinex Ultra SP-L (Novo) was then added (40 μl/3 ml extract); the solution was incubated overnight at 28 °C, being occasionally shaken. Viscosity was measured before and after treatment. The pH was adjusted to

7.0 with diluted NaOH and lectin activity was determined. Control experiments (dialysis and SDS–PAGE) revealed no evidence of proteolytic activity in the polygalacturonase preparation.

3.5. Lectin purification

3.5.1. Pigment removal and Pectinex treatment

DEAE-cellulose (0.5 g) was added to 50 ml pooled extract to further remove pigments. The suspension was stirred for 2 h at 4 °C and centrifuged at 38,000*g* for 30 min at 4 °C. Cold EtOH was slowly added to the supernatant in a cold room, with continuous stirring, up to 50% (V/V) and kept for 16 h at 4 °C. The suspension was centrifuged (38,000*g*, 30 min, 4 °C). The precipitate was suspended in a small volume of PBS-thiourea and extensively dialysed against H_2O. The viscous solution was digested (or not) with Pectinex and run, following the addition of 500 mM Tris–HCl – 1.5 M NaCl, pH 7.3, buffer (TBS × 10) (1/10 of total volume) through a DEAE-Sephadex column (2 × 15 cm) equilibrated in TBS. The non-retained peak (peak I) was either used for affinity chromatography runs or concentrated by Amicon PM 10, extensively dialysed against deionised H_2O – 5 mM thiourea and stored at −20 °C.

3.5.2. Affinity chromatography on aBSM-Sepharose 4B

Different experimental affinity chromatography conditions were assayed leading us to the method described as follows. aBSM was prepared by digesting 50 mg BSM in 0.01 N HCl at 80 °C for 1 h; the acid was removed by freeze-drying. The affinity support was prepared by coupling aBSM to BrCN-activated Sepharose 4B following the general technique described by Hermanson et al. (1992). Lectin-active fractions coming off the DEAE-Sephadex column were dialysed against PBS-5 mM thiourea, centrifuged (7000*g*, 5 min, 4 °C) and the resulting supernatant (50–100 ml) was applied to the aBSM-Sepharose 4B column (2 × 19 cm) equilibrated with PBS. The non-retained fraction was eluted with PBS and the retained peak was then eluted with 50 mM Tris-OH, pH 11.4. One millilitre fractions were received over 100 µl 2 N HCl to obtain a final pH close to neutrality and then pooled. The retained peak was extensively dialysed against 20 mM NH_4HCO_3 and freeze-dried. Protein concentration, specific agglutination activity and ELLSA assays were carried out on PBS-dissolved protein. The support's retention capacity was assessed by applying increasing amounts of pooled extracts and checking the presence of lectin in the non-retained peak.

3.5.3. DEAE-Sepharose chromatography

Remaining pigment was removed in all cases by passing the affinity purified fractions over a DEAE-Sepharose column (1 × 15 cm) equilibrated in TBS. The first peak, containing the lectin, was eluted with TBS, dialysed against 20 mM NH_4HCO_3 and freeze-dried.

3.6. Lectin detection assay

ELLSA was used for detecting lectin in crude extracts, in fractions treated and non-treated with Pectinex and purified protein, according to the described procedure (Duk et al., 1994). The plates (NUNC, F16 Maxisorp) were sensitised with aOSM (0.14 µg/ml), using biotinylated *Vicia villosa* isolectin B4 (VVB4) as control as it is specific for Tn antigen. Streptavidine-peroxidase (1.3 µg/ml) and H_2O_2-ABTS were added after suitable washes and Abs_{415} was read on a Bio-Rad ELISA autoreader. Activity (%) was calculated as (100−(Sample Abs/control Abs)×100).

3.7. Polyacrylamide gel electrophoresis

Alkaline PAGE was carried out at pH 6.8 (concentration gel), pH 8.8 (separation gel) and pH 8.3 (tank buffer). SDS–PAGE was performed in reducing and non-reducing conditions on a 10–12% separating gel (Laemmli, 1970) (10–20 µg protein/well). The behaviour of reduced and carboxymethylated protein (Yarwood, 1989) was assessed by SDS–PAGE. Tricine-PAGE was run according to Schagger and von Jagow (1987) on 10% gels; 15% separating gels were used for deglycosylated samples.

3.8. Effect of pH, temperature and cation

The effect of pH was tested (Banerjee et al., 2004) using a 2–12.5 pH range with a lectin stock solution (228 µg/ml); specific agglutination activity was determined following equilibration by dialysis (24 h) at the desired pH . The effect of temperature was assessed by erythroagglutination assay after incubating the lectin (28 µg/ml) in PBS pH 7.0 for 1 h at 25–92 °C. The effect of divalent metal ions on erythroagglutinating activity was assayed on demetallised samples by extensive dialysis against 5–100 mM $CaCl_2$ or $MnCl_2$.

3.9. Binding to glycoproteins

Glycoprotein binding was assessed by ELLSA on OSM or aOSM-coated wells (0.13 µg/ml) using biotinylated SBoL; the lectin was biotinylated (Wu et al., 1995) with two successive additions of sulfobiotin-X-NHS (Calbiochem) (2:1 w/w, 12 h interval). Excess biotin was removed by ultrafiltration with 10 kDa Nanosep filters. Biotinylated VVB4 was used as control. Binding was also assayed by affinity chromatography on Fetuin-Agarose, asialo Fetuin-Agarose obtained after neuraminidase treatment of 10 ml Fetuin-Agarose (0.15 enzyme units, 90 min, 37 °C, TBS pH 7.3) or asialo agalacto Fetuin-Agarose prepared by β-galactosidase treatment of the latter support (10 ml gel equilibrated in 0.1 M phosphate buffer, pH 7.0, 20 mg enzyme, overnight at 37 °C).

3.10. Molecular weight

Assays were carried out to determine native protein M_r on the following: (a) a Spherogel TSK 3000 SW HPLC column (15 × 300 mm) equilibrated in 10 mM phosphate buffer (pH 7.0) – 150 mM NaCl; (b) a Superose 12 column (1 × 110 cm) equilibrated in 20 mM phosphate buffer (pH 7.0) – 150 mM NaCl; (c) a Biogel P150 column (1 × 100 cm) equilibrated in 100 mM phosphate buffer (pH 7.0) – 150 mM NaCl; and (d) a Sephacryl S-200 column (1 × 100 cm) equilibrated in PBS. Bovine serum albumin (66.2 kDa), α-amylase (50 kDa), ovalbumin (45 kDa), β-lactoglobulin (36.8 kDa), myoglobin (17.2 kDa) and lysozyme (14.4 kDa) were used as standards.

Subunit M_r was determined by the Laemmli (1970) procedure; the lectin (10 μg) to which SDS was added (300 μg) was run in reducing (40 mM DTT) and non-reducing conditions. Heat-denatured protein was digested with PNGAse F (1200 mU) (Montreuil et al., 1994) and after microdialysis samples had been run on Tricine-PAGE.

Native protein molecular weight was determined by nanoflow electrospray mass spectrometry as described (Almanza et al., 2004).

3.11. Isoelectric point

The p*I* was determined with a 3.5–10 pH gradient in non-denaturing conditions following Bollag and Edelstein's procedure (1991).

3.12. Free cysteine content

The protein (5.9 mg/ml) was dissolved in 100 mM phosphate buffer (pH 7.2) – 6 M guanidine HCl – 1 mM EDTA. Free cysteine was determined as described (Creighton, 1995).

3.13. N-terminal amino acid sequence

Following SDS–PAGE, the stained protein band was transferred to PVDF (Matsudaira, 1987). Assays aimed at determining the protein's *N*-terminal sequence were carried out with an Applied Biosystems 477A sequencer.

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