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PURIFICATION OF A *N*-ACETYL-D-GALACTOSAMINE SPECIFIC LECTIN FROM THE ORCHID *LAELIA AUTUMNALIS*

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Key Word Index—*Laelia autumnalis*; Orchidaceae; plant lectin; GalNAc specific lectin; monocotyledoneae; A-blood group specific lectin; orchid.

Abstract—From the pseudobulbs of the orchid *L. autumnalis* a lectin was purified on immobilized porcine mucin with A + H blood group substance. This lectin is a dimeric glycoprotein of M_r 12 000 with an $S_{w,20}$ of 2.2, showing haemagglutinating activity directed mainly to human A_1 desialylated erythrocytes. The lectin possesses sugar specificity for *N*-acetyl-D-galactosamine and also shows high specificity for glycoproteins containing the T (galactose β1,3GAlNAcα1,0 Ser/Thr) or the Tn antigen (GalNAcα1,0 Ser/Thr).

INTRODUCTION

Lectins are proteins that specifically and reversibly bind carbohydrates and agglutinate cells [1]. Lectins are useful tools for the isolation and characterization of well-defined glycan structures and cellular subsets [2]. Many lectins have been grouped into distinct families of homologous proteins with common structural properties. The most studied plant lectins are those from the Leguminosae. However, more recently, plants from the Monocotyledones such as the orchids *Cymbidium hybridum*, *Epipactis helleborine*, *Listera ovata* [3–6] as well as some from the Gymnospermae [7] have attracted attention due to their carbohydrate binding properties, since some of them interact strongly with α-mannosyl containing structures [3, 5, 6]. In this work we describe a lectin from the pseudobulbs of the orchid *Laelia autumnalis* which exhibits a sugar and serological specificity differing from the α-mannosyl-binding lectins isolated from the leaves of other orchids [3, 4, 6].

RESULTS

Purification of Laelia autumnalis *lectin (LAL)*

From the juice obtained from the pseudobulbs of the orchid, we purified a lectin by affinity chromatography on a column containing porcine mucin with blood group substance (A + H), immobilized on Sepharose 4B. Although chromatographic procedure on purification of the lectin can be performed after $(NH_4)_2SO_4$ fractionation (at 66% final saturation), we currently performed the purification by direct application of the crude bulb extract. Elution of the purified protein was achieved by means of a pH change to 2.5 in the chromatography medium. Similar protein yields on purification were obtained using 0.2 M GalNAc instead of acid solutions. In a single purification procedure, we obtained a yield of 50% of the haemagglutinating activity and a 33-fold increase of the specific activity, as compared with the crude extract. Our results indicate that the amount of purified lectin represents only 1.5% of the total protein present in the crude extract (Table 1).

Chemical characterization

The purified LAL has a M_r of 12 000 as determined by SDS PAGE and an M_r of 25 000 as determined by non-denaturing gel electrophoresis (Fig. 1) and by ultracentrifugation on sucrose gradients (not shown); the lectin possesses a sedimentation coefficient ($S_{w,20}$) of 2.2 (not shown). The lectin is a glycoprotein that contains 2% of sugar by weight, the glycan portion presents mannose, *N*-acetyl-D-glucosamine and galactose (Table 2). As summarized in Table 3, the main amino acids found were glutamic, aspartic, serine, glycine, and alanine, and in minor proportions tyrosine, methionine, and cysteine residues. Denaturing polyacrylamide gel electrophoresis

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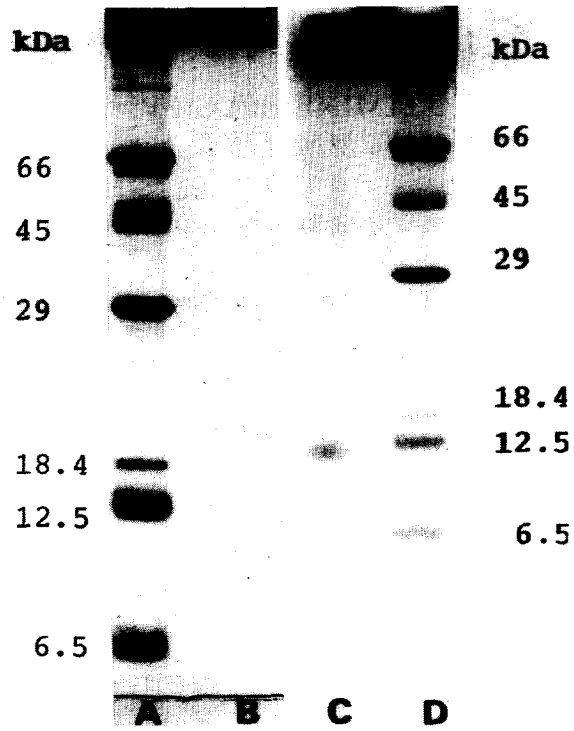

Fig. 1. Electrophoretic analysis of affinity purified *Laelia autumnalis* lectin (LAL). Lanes (a) and (b) M_r markers and LAL under non-reductive conditions; lanes (c) and (d) LAL and M_r markers in the presence of 2-mercaptoethanol and SDS, respectively. M_r markers: bovine serum albumin (66 000); ovalbumin (45 000); carbonic anhydrase (29 000); lactalbumin (18 400); cytocrome *c* (12 500); insulin (6500).

Table 1. Purification process of *Laelia autumnalis* lectin by affinity chromatography on porcine gastric mucin*

Fraction	Total protein (mg)	HAU total	Specific activity
Crude extract	192	128 000	667
Non-retained	129	0	0
Purified	3	68 000	22 700

*Data obtained from 10 g pseudobulbs. Specific activity = total haemagglutinating units (HAU)/mg protein. Haemagglutinating activity was determined in the presence of native human A_1 erythrocytes.

with a linear gradient concentration of urea (0–8 M) indicates that our preparation is homogeneous, and that the two subunits forming the active lectin are very similar (not shown).

Haemagglutinating activity

The crude extract as well as the purified *L. autumnalis* lectin showed a haemagglutinating activity four-fold higher in the presence of human A_1 erythrocytes than with O erythrocytes and eight-fold higher than with A_2 and B erythrocytes (Table 4). Neuraminidase (from *Vibrio cholerae*) or pronase treatment of erythrocytes increased the haemagglutinating titre with all the human blood groups tested; however, haemagglutinating titre is still high in the presence of A_1 erythrocytes.

Table 2. Carbohydrate composition of *Lelia autumnlis* lectin

Carbohydrate	Residues*
Galactose	1.9
Mannose	3.0
Fucose	0.2
GlcNAc	1.2

*Per three mannose residues. Arabinose, NeuAc, GalNAc or xylose were not detected.

Table 3. Amino acid composition of *Laelia autumnalis* lectin*

Amino acid	Residues/subunit
Asx	9.5
Glx	14.2
Ser	18.2
Gly	19.0
Leu	6.1
His	2.5
Phe	3.1
Arg	2.2
Lys	4.4
Thr	9.9
Ala	11.1
Pro	5.9
Tyr	0.1
Val	5.9
Met	0.7
Half Cys	0.2
Ile	3.9

*M_r 12 500.

Sugar specificity

Sugar specificity was determined by comparing the ability of sugars, glycosides, and glycoproteins to inhibit the haemagglutinating activity of LAL in the presence of neuraminidase-treated A_1 human erythrocytes. From the simple sugar tested only *N*-acetyl-D-galactosamine is able to inhibit the haemagglutinating activity of LAL (Table 5); from the glycosides tested, inhibition by the α-anomers of methyl or phenyl-galactose is twice as great as by their counterpart β-glycosides. From the glycoproteins tested, those structures possessing *O*-glycosidically linked glycans, such as fetuin, ovine and bovine submaxillary mucin, effectively inhibited the haemagglutinating activity of LAL; porcine gastric mucin, con-

taining A + H blood group substance is the most potent inhibitor of the lectin's activity. Elimination of sialic acid residues by acid hydrolysis of fetuin, ovine and bovine mucin renders those molecules to be more inhibitory than parental glycoproteins (Table 5). Other glycoproteins containing *N*-glycosidically linked glycans, such as α_1-acid glycoprotein, human serotransferrin and ovomucoid, do not show any effect on the lectin haemagglutinating activity.

Physicochemical properties

The haemagglutinating activity of LAL is not affected by treatment with EDTA/acetic acid nor by addition of Ca^{2+}, Mg^{2+} or Mn^{2+}; moreover, heat treatment above 65° for 10 min of the purified lectin abolishes the capacity of the lectin to interact with erythrocytes. The haemagglutinating activity of the lectin is stable within a pH range of 3–10; however the optimal pH of lectin activity as determined with the Universal buffer was 6.5.

Table 4. Hemagglutinating activity of *Laelia autumnalis* lectin

Blood group	Native	Pronase	Nanase
A_1	16	512	2048
A_2	2	32	64
B	2	32	128
O	4	64	512

*Titre is reported as the Inv of the last dilution with hemagglutinating activity. Nanase = neuraminidase from *V. cholerae*. Lectin concentration was 37 mg ml^{-1}.

DISCUSSION

Plant lectins represent a group of well-preserved proteins in evolution; even their sugar-binding specificities are usually well conserved [8]. Lectins have been purified from several different monocot species, which include the orchid family. Many of these lectins share a very interesting specificity for α-mannose and for high mannose structures [3, 4, 6]. In this work, we purified a lectin from the orchid *L. autumnalis*, which belongs to the epiphytae group, *Epidendroidaea*, and possesses closely related molecular characteristics with other non-epiphyphytae orchids, such as *L. ovata*, *C. hybridum*, *E. helleborine* composed by two monomeric units of an M_r of 12 500 each. Differing from other reported orchid lectins [4], *L. autumnalis* is a glycoprotein containing 2% of sugar by weight, whereas its protein component has high amounts of aspartic, glutamic, glycine, and serine residues and a minor proportion of methionine, hystidine, and cysteine. Electrophoretic analyses on urea gradients indicate that the subunits composing the lectin are similar.

LAL possesses a high capacity to agglutinate human desialylated erythrocytes from the human blood group

Table 5. Minimum concentration of carbohydrates, glycosides, glycopeptides and glycoproteins to inhibit 4 haemagglutinating units* of *Laelia autumnalis* lectin

Compound	Concentration	Relative inhibitory capacity
N-Acetyl-D-galactosamine	100 mM	1
D-Galactosamine	50 mM	2
p-Nitrophenyl-α-D-galactose	12 mM	8
p-Nitrophenyl-β-D-galactose	25 mM	4
Methyl-α-galactoside	25 mM	4
Methyl-β-galactoside	50 mM	2
Human lactoferrin	NI	—
Bovine subglandular mucin	1.0 μM	100
Asialo bovine mucin	0.2 μM	500
Porcine gastric mucin	0.05 μM	2000
Mucine submaxilar bovine	1.0 μM	100
Asialo mucin submaxilar	0.5 μM	200
Fetuin	1.0 μM	100
Asialo fetuin	0.5 μM	200
α_1-Acid glycoprotein	NI	—
Ovomucoid	NI	—
Fetuin asialo-*N*-glycopeptide	NI	—

*In the presence of human erythrocytes type A_1. Other sugars without inhibitory activity at 100 mM concentration are: D-galactose, D-glucose, D-glucosamine, *N*-acetyl-D-glucosamine, D-mannose, D-mannosamine, L- and D-fucose, L-raffinose, arabinose, melezitose, melibiose, lactose, neurmainic acid and sialyl (α2,3 or α2,6) lactose. NI = Not inhibitory at 100 μM. The relative inhibitory capacity, represents the power of inhibition when compared with *N*-acetyl-D-galactosamine.

A_1, and in minor proportion it also agglutinates A_2, O and B erythrocytes. A variety of saccharides and glycoproteins have been tested for their ability to inhibit the haemagglutinating activity of LAL. Of the monosaccharides tested, *N*-acetyl-D-galactosamine was the most potent inhibitor. α-Methyl and α-phenyl galactosides, which are better inhibitors of the lectin than the corresponding β-derivatives suggest that the lectin interacts with this anomeric configuration. Galactose does not exert any effect on the lectin's activity, and D-galactosamine is a poor inhibitor, thus indicating that for LAL the acetamido group of C_2 and the –OH on C_4 from *N*-acetyl-D-galactosamine are important determinants for the recognition mechanism. From the glycoproteins tested, fetuin, which possesses *O*- and *N*-glycosidically linked glycans [9], ovine [10] and bovine submaxillary [11] mucin are good inhibitors; asialoforms of these structures are even better inhibitors. The most potent structure recognized by LAL is the porcine gastric mucin, which exhibits A + H blood group activity.

The asialo *N*-glycopeptides from fetuin do not interact with the lectin. However, the asialo-glycopeptides (containing Gal β1,3 GalNAc α1,0 Ser/Thr) are good inhibitors. These results reinforce the idea that LAL is specific for α-GalNAc containing structures, resulting in the recognition at first instance of A_1 blood group erythrocytes [12], like the *Vicia villosa* lectin [13].

It is interesting to note that comparisons of *L. autumnalis* with other reported orchid lectins, such as *L. ovata*, *C. hybridum*, *E. helleborine*, indicate closely related similarities, specifically in M_r and dimeric conformation; however, LAL exerts different sugar-binding properties as well as blood group (serological) specificity [4, 6]. The lectins from the group of orchids seem to possess some similarities on their combining site for α-anomeric configurations, although differences on the specific-binding capacity could be due to differences in the amino acid composition of the active site, as observed with some lectins from the leguminous group [8]. Although more studies are needed, the possibility that the differences found in the sugar specificity of these two groups of orchid lectins could be due to adaptation mechanisms should not be ruled out.

We conclude that the lectin from *L. autumnalis* could be considered a good tool for the study of A_1 blood group substances. Moreover, the lectins from the Orchidaceae could be considered as molecular markers in the evolution of this group of plants, which contains over 10000 representative organisms [14].

EXPERIMENTAL

Material. Laelia autumnalis (Llave & Lex.) orchids were collected in Tetela del Volcán (State of Morelos, Mexico) and classified at the Centro de Investigaciones Biológicas (Universidad del Estado de Morelos, Cuernavaca, Morelos State, Mexico). All the carbohydrates, glycosides, glycoproteins and enzymes used in this study were from Sigma (Sigma Chemical Co, St Louis, MO, U.S.A.).

Purification of the lectin. The juice of the pseudobulbs was obtained with a food processor, and centrifuged at 7000 *g* for 20 min to eliminate all the particulate material. The lectin was purified by affinity chromatography on a column (5 × 1 cm i.d.) containing porcine gastric mucin with A + H blood group substance activity coupled to Sepharose 4 B (Sigma). The clear juice (30 ml) was applied to the column, which was previously equilibrated with PBS (phosphate-buffered saline (PBS), 0.01 M NaPi, 0.14 M NaCl, pH 7.4) at a flow rate of 15 ml‖h^{-1}. Unretained material was eluted with PBS until A_{280} was below 0.01, then the lectin was eluted by adding 3% HOAc in H_2O. Fractions of 2 ml were monitored at A_{280} below 0.01. Fractions (2 ml) were monitored at A_{280} and dialysed against PBS before testing the haemagglutinating activity in the presence of human A_1 erythrocytes.

Analytical methods. Protein concn was determined by the method of ref. [15], using bovine serum albumin as a standard. Total carbohydrate concn was determined by the PhOH–H_2SO_4 method [16], using lactose as standard. Amino acid composition was determined in a Beckman 119-CL amino acid analyser using 100 mg of purified lectin in 30 ml of 0.3% PhOH to prevent degradation of tyrosine residues, followed by hydrolysis for 24 hr under vacuum with 2 ml of 6 M HCl at 110° in sealed containers. The quantitative composition of sugar was determined by gas chromatography as trifluoroacetyl alditol derivatives after methanolysis, using meso-inositol as internal standard; per-*O*-trimethylsilylated methyl glycosides (after *N*-re-acetylation) were analysed in a Varian 2100 GC using a 5% silicone OV 201 column (25 × 0.32 mm i.d.) [17].

Molecular weight and homogeneity of the purified lectin were determined by SDS–PAGE using 5–20% gradient gels in the discontinuous buffer system of ref. [18]. Gels were silver-stained according to the procedure described in ref. [19]. The ultracentrifugation experiments in 5–25% linear sucrose gradients in H_2O were performed in a Beckman LS-65 ultracentrifuge. The lectin sedimentation coefficient ($S_{w,20}$) was determined according to ref. [20], the native lectin M_r was also determined by ultracentrifugation according to ref. [21].

Haemagglutinating activity. Human erythrocytes type A_1, A_2, B, and O from healthy human donors were obtained from the Central Blood Bank IMSS, Mexico. Haemagglutinating activity of the crude extract and the purified lectin were assayed in microtitre U-plates (Nunc, Denmark) according to the two-fold serial dilution procedure [22]. The agglutinating activity was tested with either a 2% (v/v) untreated human erythrocytes suspension in PBS, with neuraminidase treated (0.1 U of *Vibrio cholerae* neuraminidase per 0.5 ml of packed erythrocytes at 37° for 30 min [23]), or pronase-treated (100 mg of *Streptomyces griseus* pronase per 0.5 ml of packed erythrocytes at 37° for 30 min) erythrocytes [24]. Titre is reported as the inverse of the last dilution with positive agglutination.

Sugar specificity. Sugar specificity was determined comparing the inhibitory activity of various sugars, gly-

coproteins or their derived glycopeptides and glycans on the haemagglutinating activity induced by the lectin against neuraminidase treated human A_1 erythrocytes. Results are expressed as the minimal concn required to completely inhibit four haemagglutinating doses (titre = 4). The glycoprotein molar concn was determined according to the M_r with desialylated glycoproteins we subtracted the number of sialic acid molecules released from the native protein. The molar concn of glycans and glycopeptides was calculated on the basis of their oligosaccharide content as determined by GC. Molar concn of porcine gastric mucin assumed an M_r of 1 000 000.

Preparation of fetuin glycopeptides. Fetuin glycopeptides were obtained by pronase digestion (1 mg of enzyme per 10 mg of glycoprotein incubated for 48 hr at 37°). The *O*- and *N*-glycosyl peptides were fractionated by gel-filtration on a column (100 × 1.6 cm i.d.) containing Bio-Gel P-4 equilibrated with 0.05 M pyridine acetate, pH 4.5, as described previously [25]. Glycoproteins, glycopeptides or glycans were desialylated by incubation at 100° for 1 hr in the presence of 0.02 M H_2SO_4, as described in ref. [9], and desalted on a Bio-Gel P-2 column (60 × 2 cm i.d.) previously equilibrated with H_2O.

Physicochemical parameters. Temp. effect and divalent metals dependence. Temperature stability of the lectin was determined after incubation at 60–100°, for several time intervals. The effect of pH on the haemagglutination induced by the LAL was determined directly, using either 1% glutaraldehyde treated erythrocytes in the pH range 3–9, or indirectly, by an adsorption–desorption procedure using a stroma column [25]. The buffer used was 0.2 M glycine adjusted to the desired pH value with 0.2 M HCl or 0.2 M NaOH (Universal Buffer) [26]. The effect of divalent metals on the haemagglutining capacity of the lectin was estimated by mixing the metal soln (5 mM $CaCl_2$, $MnCl_2$, $MgCl_2$ in PBS) with a lectin soln previously dialysed against 0.1 EDTA in PBS. Agglutination assay was conducted as indicated above.

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