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Lectin from *Phaseolus acutifolius var. escumite*: Chemical Characterization, Sugar Specificity, and Effect on Human T-Lymphocytes

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Purification of the lectin from *Phaseolus acutifolius var. escumite* was achieved by affinity chromatography on a column containing glutaraldehyzed membranes from blood group O erythrocytes. The lectin is a tetrameric glycoprotein of 121 kDa with 10% of sugar by weight composed by four subunits of 30 kDa as determined by SDS-PAGE. The lectin is composed of four isolectins as determined by ion-exchange chromatography on a mono-S column. The lectin and its isolectins showed identical NH2 terminal residues (ANDLSFNFQR FNETN) with homology to the PHA leucoagglutinin-precursor. Peptide mass fingerprint from each lectin isoform determined from tryptic peptides by MALDI-TOF (matrix assisted laser desorption ionization-time-of-flight) showed differences among subunits, thus suggesting microheterogeneity in their amino acid sequences or different glycosylation patterns. The lectin and its four isolectins agglutinated erythrocytes without serological specificity and showed mitogenic activity on human leukocytes; moreover, the main effect was rather toward CD8+ than to CD4+ human peripheral lymphocytes. The lectin from escumite was not inhibitable by simple sugars; however, the specificity of the lectin and its isoforms was mainly addressed toward galactose residues present in bi- or triantennary *N*-acetyllactosamine-type glycans.

KEYWORDS: ***Phaseolus acutifolius var. escumite*; lectins; glycoproteins; lectin sugar specificity; mitogenic lectin**

INTRODUCTION

Legume lectins are of great interest due to their use as tools in the study of the physiology of the nutrition and in cellular immunology (*1*−*3*). Particularly lectins from the *Phaseolus* genus and from a number of cultivars of the common bush bean *Phaseolus vulgaris* have been isolated (*4*−*6*). Some varieties of these lectins correspond to a mixture of five tetramers derived from the combination of two subunits, namely, erythroagglutinin (E-PHA) and leucoagglutinin (L-PHA), which are noncovalently associated (*6*−*8*). Other varieties are constituted by three, two, or one lectin subunit (*7*, *8*). Plant lectins have been used to identify several distinct lymphocyte subpopulations in normal or in lymphoproliferative disorders; besides, PHA can inhibit blastogenic transformation and mediate apoptosis of B- and T-cell tumor lines (*9*). Phytohemagglutinin treatments can also impede tumor growth in mice while showing no toxic side effects in this animal model, suggesting that specificity for specific sugar-ligands for this group of lectins plays a relevant role in normal and tumor cells (*9*). Antifungal and antiviral activities have been also demonstrated in lectins from the *Phaseolus* genus (*10*).

The leucoagglutinating subunit from *P. vulgaris* var. red kidney bean binds lymphocytes with a high degree of specificity and induces blastogenic activity in these cells. The interaction of the lectin with cells from the immune system induces other effects aside from mitogenic stimulation (*11*), such as immunosuppression (*5*, *12*) or induction of skin allograft survival (*13*). The biological quality of *Phaseolus acutifolius* seeds makes them a potentially good protein source and supports their great potential as a donor of genes to achieve better bean varieties

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(*14*). The presence of antinutritional components and cytotoxic effects of *P. acutifolius* bean extracts have been reported, and these effects have been attributed to their high content of trypsin inhibitors, tannins, and lectins (*15*). Previous works have identified a tetrameric lectin isolated by affinity chromatography from *P. acutifolius* seed extracts, which shows hemagglutinating and mitogenic activity and also delays development of bruchid beetles (*Acanthoscelides obtectus*) (*16, 17*) and with toxic effects on rodents at doses as high as 1.1 g/kg weight (*18*). This work was aimed at purifying and characterizing a lectin from *Phaseolus acutifolius* beans that possesses both erythroagglutinating and mitogenic properties.

MATERIALS AND METHODS

Materials. *Phaseolus acutifolius* var. escumite seeds were obtained in a local market and classified at the Centro de Investigaciones Biológicas, Universidad de Morelos, Morelos, Mexico. Sialidase (from *Vibrio cholerae*, fraction V EC 3.2.1.18), pig stomach mucin grade II, fetuin grade II, hen ovomucoid, β-galactosidase from *Canavalia ensiformis* (jack bean), *p*-formaldehyde, fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin, β-mercaptoethanol, sodium pyruvate, RPMI-1640 culture medium, trypan blue dye, and all sugars were purchased from Sigma Fine Chemicals (St Louis, MO). Phycoerythrin (PE)-labeled monoclonal antibodies (mAbs) against human CD8, Quantum Red (QR) and fluorescein isothiocyanate (FITC)-labeled mAb against human CD4, carboxyfluorescein diacetate succinimidyl-ester (CFSE) were from Molecular Probes (Eugene, OR).

Lectin Extraction and Purification. *P. acutifolius* var. escumite lectin (EsL) was purified from saline extracts of the ground seeds by affinity chromatography on a column containing glutaraldehyzed human erythrocytes membranes (stroma). Erythrocytes stroma from adult blood group O was obtained by lysis of red blood cells. The membranes were fixed with 1% glutaraldehyde in PBS (0.01 M Na_2PO_4, 0.15 M NaCl, pH 7.4) overnight at 4 °C, and then washed with distilled water. Erythrocytes stroma was physically entrapped in a 25 × 1.5 cm column containing Sephadex G-25 (Pharmacia Fine Chem., Uppsala, Sweden) as described (*19*). The column was equilibrated with PBS at a flow rate of 40 mL/h. *P. acutifolius* extract (160 mg protein) was applied to the column, and nonretained material was eluted with PBS until the A_{280} of 1 mL collected fractions was below 0.01. The lectin (EsL) was eluted from the column with 3% acetic acid, and the absorbance at 280 nm was determined in 2 mL eluted fractions; the pH of each fraction was adjusted with PBS before determination of the hemagglutinating activity of each collected fraction in the presence of 2% human group O erythrocytes.

Analytical Methods. The protein concentration was determined by the method of Bradford (*20*) with Coomassie blue R-250, using bovine serum albumin as the standard. The carbohydrate concentration of the purified lectin was determined by the phenol-sulfuric method (*21*), using lactose as the standard. Amino acid composition of EsL was determined as follows: 100 μg of EsL was hydrolyzed under vacuum with 2 mL of 6 M HCl at 110 °C in sealed tubes for 72 h. The samples were analyzed on an automatic amino acid analyzer Durrum 500, using norleucine as the internal standard (*22*). Carbohydrate composition of the purified lectin was determined with the heptafluorobutyrate derivatives of *O*-methyl-glycosides, obtained after methanolysis in 0.5 M methanol-HCl for 24 h at 80 °C; lysine was used as internal standard. The samples were analyzed by gas-chromatography using a capillary column (25 × 0.32 mm) of 5% Silicone OV 210, (Applied Science Lab., Buffalo, NY) in a Varian 2100 gas chromatograph (Orsay, France) (*23*).

Determination of Molecular Mass. Molecular mass of native purified EsL was determined by gel filtration chromatography on a column (100 × 1.6 cm) containing Sephacryl S-300 (Pharmacia, Uppsala, Sweden), equilibrated with PBS at a flow rate of 12 mL/h. Absorbance at 280 nm was determined in 1 mL eluted fractions, and hemagglutinating activity was assessed in the presence of 2% human group O erythrocytes. Relative molecular mass of the lectin was obtained by comparing the elution profile with molecular weight standards (Sigma). The molecular mass and the homogeneity of the purified lectin and of its isolectins were evaluated in 10% polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% sodium dodecyl sulfate (SDS), with the Laemmli buffer system (*24*); gels were stained with 0.1% Coomassie brilliant blue G250 (Sigma).

Identification of EsL Isolectins by Ionic Exchange Chromatography. The affinity purified lectin (300 μg/100 μL) was applied to a mono-S pre-packed HR column 5/20 mm (Pharmacia, Uppsala, Sweden), equilibrated previously with 5 mM acetate buffer, pH 4.5, at a flow rate of 1 mL/min with a maximal pressure of 40 bar, in a 60 min program using an FPLC System (Pharmacia, Uppsala, Sweden) (*25*). Lectin isoforms were eluted from the column with a 0−1 M NaCl stepwise gradient in acetate buffer. Fractions (1 mL) were collected, and the absorbance was monitored at 280 nm. Each eluted peak was dialyzed against distilled water before lyophilization for further analysis.

Amino Acid Sequence and MALDI-TOF Analysis. The amino acid sequence was determined on the purified EsL, electrophoresed on SDS-PAGE, and transferred to a PVDF membrane; the band was excised from the blot and sequenced with a Beckman model LF3000 protein sequencer (Fullerton, CA). Peptide mass fingerprint of the EsL and of its isolectins was determined by MALDI-TOF (matrix assisted laser desorption ionization-time of flight) on peptide fragments obtained by trypsin digestion of the purified lectin and of its isoforms; it was performed after electrophoresis, and bands were excised with a scalpel and analyzed as follows: The band containing 200 pM of protein was digested with 0.5 μg trypsin in 500 mL ammonium bicarbonate, pH 8.0, at 37 °C, for 24 h. The reaction was inhibited by storing at 4 °C. Then, the enzyme digest was evaporated to dryness using a Gyrovap (Howe, London). Samples were prepared by mixing directly onto the target 1 μL of the reaction products (containing 50 pM) and 1 μL of a 2,5-dihydroxybenzoic acid matrix (12 mg/mL in CH_3OH/H_2O, 70:30, v/v) and then allowing the mixture to crystallize at room temperature (*26*). Positive ions of the peptides were measured by MALDI-TOF on a Vision 2000 time-of-flight mass spectrometer (Finnigan MAT, Bremen, Germany) equipped with a 337 nm UV laser. The mass spectra were acquired in reflectron mode under 8 keV acceleration voltage and positive detection. Control assays were performed using trypsin alone to identify self-digested peptide mass and with angiotensin-I as standard (Mr 1296.7). The mass of $[M+H]^+$ ions from peptides produced by tryptic digestion were compared with that of peptides obtained from NCBInr (ProFound 2004/06/01) database.

Hemagglutinating Activity. Erythrocytes of several species, were collected in a sterile Elsevier's solution (100 mm glucose, 20 mm NaCl, and 30 mm sodium citrate, pH 7.2) and washed four times through centrifugation (800 *g* for 10 min) with PBS. Erythrocytes from rabbit (New Zealand), mice (CD-1), and rat (Wistar) were obtained from the animal facilities at the Faculty of Medicine, UNAM, Mexico; human A, B, and O erythrocytes were obtained from healthy donors. Hemagglutinating activity of EsL was assayed in microtiter U plates (NUNC, Denmark) by 2-fold serial dilution. The agglutinating activity was tested with 2% erythrocyte suspension in PBS. Hemagglutinating activity is reported as the reciprocal of the last dilution showing visible hemagglutinating activity. Hemagglutination assays were also performed in the presence of erythrocytes previously treated with 0.1 U of *Vibrio cholerae* sialidase per 0.5 mL of packed erythrocytes at 37 °C for 30 min.

Cell Proliferation Assay. Peripheral blood mononuclear cells (PBMC) from human healthy donors were isolated from 10 mL of heparinized whole blood samples diluted 1:1 in PBS. This mixture was overlaid onto 5 mL of hystopaque (1.077 density) in centrifuge tubes and centrifuged at 800*g* at room temperature (22 ± 3 °C) for 30 min. PBMCs were collected from the interface layer, washed twice in PBS, and counted in a hemocytometer, assessing their viability by trypan blue dye exclusion. PBMCs were stained for flow cytometry with carboxyfluorescein diacetate succinimidyl-ester (CFSE) (*27*). Briefly, 1 mL of PBMC at a density of 10^7 cells/mL of RPMI medium was incubated with 15 μL of 0.5 mM CFSE, for 10 min at room temperature in the dark. After incubation, 8 mL of complete culture medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 M β-mercaptoethanol, 0.1 M sodium pyruvate, 100 IU/mL penicillin, and 100 μg/mL streptomycin, was added to the cells. Subsequently, cells were centrifuged and suspended in 1 mL of

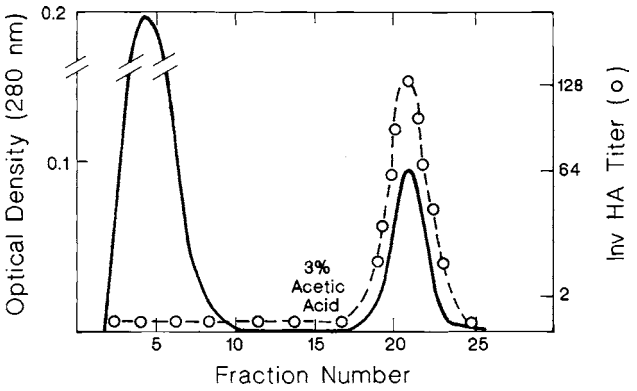

Figure 1. Purification of the lectin from *Phaseolus acutifolius* var. escumite (EsL) by affinity chromatography. *P. acutifolius* extract in PBS was applied onto a column (25 × 1.5 cm) containing glutaraldehyde fixed human type O erythrocytes stroma, physically entrapped in Sephadex G-25 equilibrated in PBS. Unbound material was eluted with PBS, and the retained EsL was eluted with 3% acetic acid in water. Absorbance at 280 nm and hemagglutinating activity with 2% human erythrocytes type O in PBS were measured after dialysis of each 2-mL fraction against PBS.

supplemented culture medium. CFSE-treated PBMCs were seeded into 96-well culture plates (Nalgene Nunc International, Denmark) at 2 × 10^5 cells per well in 200 μL of supplemented RPMI-1640 culture medium at 37 °C in a 5% CO_2 humidified atmosphere. Proliferation assays were performed on cells incubated in the presence of different concentrations of EsL, of its isolectins, or of Con A in RPMI as mitogenic control. After stimulation, the cells were harvested, stained with murine PE-conjugated anti-CD4 or CD8 monoclonal antibodies, and analyzed on a FACSCalibur equipped with CellQuest software (Becton-Dickinson, San Jose, CA). Blast cells were gated by forward scatter (FSC) and side scatter (SSC) characteristics. Cell proliferation was evaluated by reduction in the fluorescence intensity of CFSE.

Sugar Specificity. Glycoproteins were desialylated by incubation at 100 °C for 1 h with 0.02 N sulfuric acid (*28*), and desalted on a Bio-Gel P-2 column (2 × 60 cm) equilibrated with water. β-Galactosidase digestion of calf asialo-fetuin, human asialo-IgA, and asialo-α_1-acid glycoprotein was carried out at 37 °C for 8 h with 10 mU of Jack bean β-galactosidase for each 100 μM of glycan as described by Spik et al. (*29*). Then the asialo-agalacto-glycoproteins were desalted on a Bio-Gel P2 column equilibrated with water and lyophilized until use. The sugar specificity of EsL was determined by comparing the inhibitory activity of various sugars and glycoproteins, with known sugar structures, on the agglutination induced by the lectin against human type O erythrocytes. Results were expressed as minimal concentration required to completely inhibit four hemagglutinating doses (Titer=4).

RESULTS

Lectin Purification. The lectin from the saline extract from *P. acutifolius* var. escumite (EsL) was purified on a column with Sephadex G-25 containing glutaraldehyzed stroma from human type O erythrocytes. The nonretained fraction was eluted with PBS and showed no agglutinant activity in the presence of any of the tested erythrocytes. The lectin was eluted with 3% acetic acid, and the obtained fractions showed hemagglutinating activity (**Figure 1**). By this procedure, the lectin increased 166 times its specific activity when compared with the specific activity of the crude extract. The EsL's concentration represented 1.8% of the total protein from the crude extract (**Table 1**).

Chemical Characterization of EsL. The purified lectin, in nondenaturing conditions, is a protein of 121 kDa as demonstrated by gel filtration chromatography (**Figure 2A**); SDS-PAGE revealed EsL to be composed by 30 kDa subunits (**Figure 2B**). EsL is mainly composed by aspartic and glutamic acid, serine and glycine; in a minor proportion, methionine and tyrosine were identified, and it lacks cysteine residues (**Table 2**). EsL contained 10% of carbohydrates by weight, and as determined by gas chromatography analysis, it is mainly composed by Man, GlcNAc, and Gal; Xyl was identified in a lower proportion (**Table 3**).

Table 1. Purification of *Phaseolus vulgaris* var. *escumite* Lectin (EsL) and Isolectins[a]

fraction	protein (mg)	hemagglutinating specific activity[b]	purification index
crude extract	1728	37	1
affinity chromatography			
non retained	1640	0.0	
EsL	31.1	6164	166
ion exchange chromatography			
F I	2.8	4410	119
F II	5.4	6940	187
F III	3.1	4829	130
F IV	5.0	5410	146

[a] From 10 g of dry escumite flour. [b] Hemagglutinating activity/mg of protein determined in the presence of human erythrocytes type O.

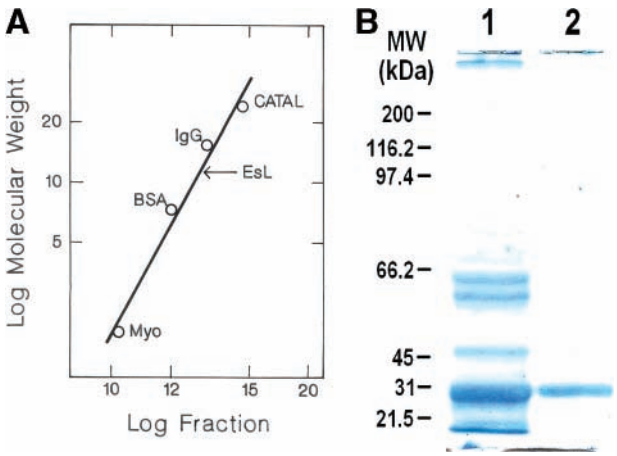

Figure 2. (**A**) Molecular mass determination of purified EsL by gel filtration chromatography on Sephacryl S-300. Calibration curve was obtained by plotting marker proteins (Catalase 240 000; IgG 150 000; bovine serum albumin 68 000; myoglobin 18 000). (**B**). SDS electrophoresis of the purified *Phaseolus acutifolius var*. escumite lectin (1) 75 μg of escumite crude extract, and (2) the affinity purified escumite (20 μg) lectin. The molecular weight standards used were myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase B (97.4), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and trypsin inhibitor (21.5 kDa).

EsL seems to be composed of a mixture of isolectins that were resolved into four fractions by ion exchange chromatography on a mono-S column (**Figure 3**). The four fractions isolated (termed FI, FII, FIII, and FIV) contained 70% of the hemagglutinating activity from EsL (**Figure 3** and **Table 1**). The EsL isolectins are tetrameric proteins derived from the association of 30 kDa subunits, as determined in the SDS-polyacrylamide gel electrophoresis (not shown).

The amino acid sequencing of EsL or of its separated isolectins showed an identical NH2-terminal sequence ANDLS-FNFQR FNETN. The peptide mass fingerprint was determined on EsL and its isoforms after trypsin digestion, by MALDI-TOF. Digestion of the lectin and its isolectins yielded peptides detectable by MALDI-TOF within the *m*/*z* range of 768.36−

Table 2. Amino Acid Composition of *Phaseolus acutifolius* var. Escumite Lectin[a]

amino acid	residues %	res/mol
Asx	13.3	146.5
Glx	8.0	81.6
Ser	12.5	139.4
Gly	11.9	131.0
His	1.5	16.0
Arg	3.0	33.1
Thr	7.3	79.2
Ala	6.7	71.2
Pro	3.9	42.1
Tyr	1.4	16.1
Val	6.1	68.2
Met	0.4	4.4
Cys	0.0	0.0
Ile	4.7	47.2
Leu	8.8	91.9
Phe	5.1	60.0
Lys	6.5	67.9

[a] Calculated on the basis of 121 kDa and considering 10% of sugar by weight.

Table 3. Carbohydrate Composition of *Phaseolus acutifolius* var. Escumite Lectin[a]

carbohydrate	residues %	res/mol
galactose	22.3	14.9
mannose	39.1	26.3
GlcNAc	30.5	16.6
xylose	7.9	5.7

[a] Calculated on the basis of 121 kDa, and considering 10% of sugar by weight. Fucose, sialic acid, rhamnose, glucuronic acid, or GalNAc were not detected.

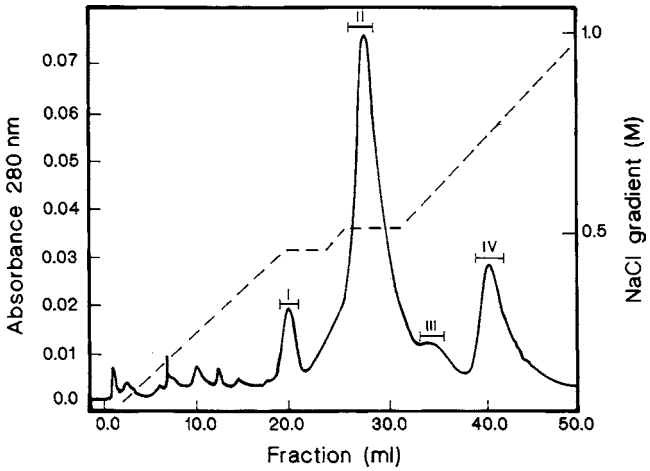

Figure 3. Purification of the *P. acutifolius* var. escumite isolectins by ion exchange chromatography. The mono S column was equilibrated with 5 mM sodium citrate, pH 4.5, and the isolectins eluted with a discontinuous NaCl gradient (0–1 M) (dotted line). Absorbance at 280 nm was measured in each 0.5 mL fraction (continuous line). The bar under roman number indicates the fractions considered for each isolectin.

3379.76. Peptides comparison with the NCBInr database revealed the EsL isoforms peptides matched with the precursor of the *P. vulgaris* red kidney bean L-subunit and covered 21, 23, 27, and 31% of this protein for EsL I, II, III, and IV isolectins, respectively, and analysis of the peptides obtained from the 30 kDa EsL showed 31% identity with the L-precursor of PHA (**Table 4**).

Hemagglutinating Activity of EsL. Neither EsL nor its isoforms revealed specificity for any of the human blood groups since they agglutinated human type A, B, and O erythrocytes at 2.1 μg/mL. The minimal agglutinating doses were 3.0, 2.6, 2.1, and 2.0 μg/mL, for EsL and isoforms I, II, III, and IV, respectively. Similarly, EsL or its isolectins agglutinated equally well rabbit, mice, or rat erythrocytes. Sialidase treatment of the human or animal erythrocytes increased the lectin agglutinating activity 6-fold.

Table 4. Predicted Amino Acid Sequence of *Phaseolus acutifolius* Var. Escumite Lectin and Isolectins Determined from Tryptic Peptides by MALDI-TOF

m/z	EsL isolectin	residue no[a]	sequence
1799.870	I, II, III, IV	56–72	LTNLNDNGE PTLSSLGR
2218.197	III, IV	102–124	VPNNAGPADG LAFALVPVGSKPK
1634.783	I, III	140–153	AHTVAVEFD TLYNR
1094.605	I, II, IV	161–170	HIGIDVNSIK
2304.077	II, IV	140–158	AHTVAVEFDT LYNRDWDPR
1766.815	II, III, IV	240–255	GNVETNDLL SWSFASK
2218.197	I, II, III, IV	256–276	LSDGTTSEGL NLANFVLNQIL

[a] Amino acid residues are numbered according to the determined sequence of the leucoagglutinating PHA precursor. Indicated peptides were identified in the analysis of EsL; those peptides identified in the EsL I, II, III, or IV isolectins obtained from ion-exchange chromatography are also indicated.

Effect of EsL on Human Leucocytes. EsL and its four isoforms showed mitogenic activity toward human peripheral lymphocytes, and an optimal concentration of 15 μg/mL was determined through dose response kinetics for EsL; optimal concentrations to induce a similar mitogenic effect as EsL were 9.9, 12, 12.8, and 13.1 μg/mL for the purified isoforms I, II, II, and IV, respectively. EsL induced cell proliferation of both CD4+ and CD8+ lymphocytes; however, proliferation of CD8+ was almost 3-fold greater than that of CD4+ cells (**Figure 4**); similarly, the four isolectins showed a higher capacity to stimulate CD8+ lymphocytes (not shown).

Sugar Specificity of EsL. Monosaccharides were not recognized by the EsL or by its isoforms; therefore, the carbohydrate specificity of the lectin was determined by comparing the inhibitory ability of glycoproteins with known oligosaccharide structures on the lectin agglutinating activity. As shown in **Table 5**, calf fetuin, human serum α_1-acid glycoprotein, and human IgA, in minor proportion, were effective inhibitors, whereas hen ovomucoid, human and bovine lactoferrin, and pig stomach mucin, at concentrations as high as 100 μM, failed to inhibit the agglutinating activity. Desialylated glycopeptides from fetuin, IgA, and α_1-acid glycoprotein inhibited the isolectins' hemagglutinating activity, but the removal of galactose residues from the asialo-forms of fetuin, α_1-acid glycoprotein, and IgA by β-galactosidase treatment abolished the inhibitory capacity of these glycoproteins (**Table 5**), indicating that galactose residues are necessary to inhibit the hemagglutinating effect of EsL. Similarly to EsL, the isoforms of the lectin were inhibited by glycoproteins and their asialo forms but not by asialo-agalacto-glycoproteins (not shown).

DISCUSSION

Lectins from leguminous plants constitute a large family of homologous proteins displaying remarkable divergence in their carbohydrate specificity. Elucidation of the mechanism by which

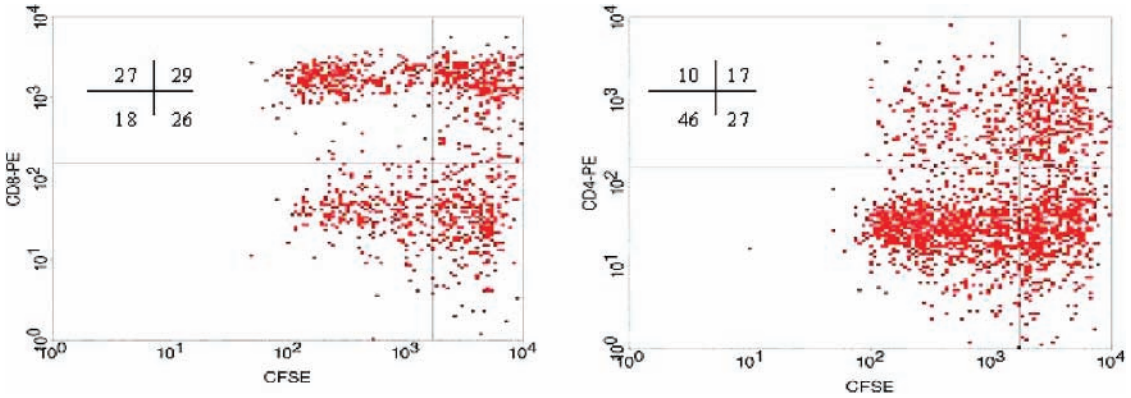

Figure 4. Proliferation effect of EsL on human lymphocytes. CFSE labeled human peripheral blood mononuclear cells were stimulated with 15 μg/mL of EsL for 72 h. The cells were harvested and stained with monoclonal antibodies PE-labeled anti CD8 (left) and -anti CD4 (right) and analyzed by flow cytometry. The inner numbers represent the cell percentage of each quadrant in the density blot. Assays were performed by triplicate, and standard error of the mean was never higher than 10%. Representative result from one assay out of five.

Table 5. Minimum Concentration of Glycoproteins Necessary to Inhibit the Hemagglutinating Activity of EsL[a]

glycoprotein	concentration (μM)
human α_1-acid glycoprotein	2.7
asialo α_1-acid glycoprotein	0.8
asialo-agalacto α_1-acid glycoprotein	N. I.
IgA	4.8
asialo-IgA	2.5
asialo-agalacto IgA	N. I.
calf fetuin	1.3
asialo-fetuin	0. 2
asialo-agalacto-fetuin	N. I.

[a] The lectin concentration was adjusted to 3 μg/mL, and the hemagglutinating activity was tested in presence of human group O erythrocytes. Sugars with noninhibitory activity at 200 mM (N. I.) included: Glc, Man, Gal, L- and D-Fuc, GlcNAc, GalNAc, NeuAc, and lactose. Glycoproteins with noninhibitory activity at 100 μM were bovine lactoferrin, human serotransferrin and lactoferrin, hen ovomucoid, or pig stomach mucin.

these lectins can possess such a broad range of binding specificities while maintaining a strikingly similar three-dimensional monomer structure will be the key to understand the essence of carbohydrate−protein interactions (*30*, *31*). In this work, we report the isolation and characterization of EsL, a lectin from *P. acutifolius* var. escumite, which is a tetrameric glycoprotein of 30 kDa per subunit. Lectins from the common bush bean *Phaseolus vulgaris* have erythroagglutinating and mitogenic activities. These lectins correspond to a mixture of five tetramers derived from the combination of two subunits: erythroagglutinin and leucoagglutinin, which are noncovalently associated (*5*). Other varieties are constituted by three, two, or one lectin subunit, and their biological activity is well correlated with the type of subunit present in such varieties and can show or not erythro- and leuco-agglutinating effects (*6*, *7*). Our results indicate that EsL is composed by four isolectins, formed by tetramers derived from the assembly of subunits that possess identical terminal amino acid residues. Peptide mass finger print of each isolectin by MALDI-TOF indicated some differences in the tryptic pattern among isolectins that could be the result of a differential glycosylation pattern among isoforms. EsL is a glycoprotein with 10% of sugars by weight and seems to contain N-glycosidically linked oligosaccharides, thus, suggesting that each lectin isoform represents the allelomorphic expression of the same gene with a different glycosylation pattern, a hypothesis that should not be ruled out (*32*). The erythroagglutinating subunit of PHA from *P. vulgaris* agglutinates erythrocytes but not lymphocytes (*2*, *3*); interestingly, EsL and its four isolectins showed both erythroagglutinating and mitogenic capacity on human leukocytes. Although EsL agglutinates human erythrocytes irrespective of their blood group, the capacity of the lectin to induce blastogenic properties seems to be addressed mainly to CD8+ rather than to CD4+ human T-lymphocytes. These results agree with those reported by Rutella et al., who identified that CD8+ cells exhibited a greater activation response to PHA (*33*).

The *P. vulgaris and P. coccineus* lectins recognize N-glycosidically linked oligosaccharides with a minimal octasaccharide structure with the sequence Gal(β1−4)GlcNAc(β1−2)Man(α1−6)[GlcNAc(α 1−2)Man(α1−3)]Man(α1−4) (*34*−*38*). Lectins of 21 and 30 kDa have been described in *P. acutifolius* var. latifolius and in tepary bean (*P. acutifolius*) (*17*), and these lectins, just like the *P. acutifolius var. escumite* lectin, are not inhibitable by monosaccharides; moreover, our results showed that EsL is specific for glycoproteins that possess bi- and triantennary N-glycosidically linked glycans of the *N*-acetyllactosaminic type, such as fetuin (*26*, *39*), human IgA (*40*), and α_1-acid glycoprotein (*41*). Fetuin and α_1-acid glycoprotein are powerful inhibitors of EsL, than human IgA. Fetuin and the α_1-acid glycoprotein showed important microheterogeneity of different bi-, tri- and tetra-antennary *N*-acetyllactosamine-type glycans (*39*, *41*). The importance of galactose in the interaction with fetuin, α_1-acid glycoprotein, and IgA is suggested by the lack of affinity of EsL with structures devoid of this oligosaccharide determinant, such as bovine lactoferrin and hen ovomucoid (*42*) or asialo-agalacto- α_1-acid glycoprotein and fetuin, nor with porcine stomach mucin, which contains *O*-glycosidically (Galβ1,3GalNAc) linked glycans (*32*) indicating that Gal(β1−3 or 4)GlcNAc(α 1−2)Man sugar sequence is essential for the binding. Our results suggest that EsL is a useful tool for immunological studies due to its capacity to activate mainly CD8+ T cells and could also be useful for the study of lactosamine type *N*-glycans, since the carbohydrate moieties of cell surface glycoconjugates are known to play important roles in cell adhesion (*43*, *44*) and have been shown to be a marker of tumor progression (*45*, *46*).

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LITERATURE CITED

(1) Sharon, N., Lis, H., Eds.; *Lectins*; Kluwer Academic Publishers: The Netherlands, 2003.

(2) Casaubon-Huguenin, M. T.; Ávila, E.; Vazquez, C.; Trigo, F.; Lascurain, R.; Zenteno, E. The effect of raw full-fat soybean and its lectin in nutrition and pigmentation of broilers. *J. Agric. Food Chem.* **2004**, *52*, 5702−5708.

(3) Liener, I. E. Nutritional significance of lectins in diet. In *The lectins, properties, functions and applications in Biology and Medicine*; Liener, I. E., Sharon N., Goldstein, I. J., Eds.; Academic Press: New York, 1986.

(4) Felsted, R. L.; Li, J.; Pokrywka, G.; Egorin, M. J.; Spiegel, J. Comparison of *Phaseolus vulgaris* cultivars on the basis of isolectin differences. *Int. J. Biochem.* **1981**, *13*, 549−557.

(5) Vargas-Albores, F.; Hernández, J.; Córdoba, F.; Zenteno, E. Isolation of an immunosuppressive lectin from *Phaseolus vulgaris* cv. cacahuate using stroma. *Prep. Biochem.* **1993**, *23*, 473−483.

(6) Osborn, T. C.; Ausloos, K. A.; Brown, J. W. S.; Bliss, F. A. Bean lectins. III. Evidence for greater complexity in the structural model of *Phaseolus vulgaris* lectin. *Plant Sci. Lett.* **1983**, *31* 193−203.

(7) Felsted, R. L.; Egorin, M. J.; Leavitt, R. D.; Bachur, N. R. Recombinations of subunits of *Phaseolus vulgaris* isolectins. *J. Biol. Chem.* **1977**, *252*, 2967−2971.

(8) Brown, J. W. S.; Osborn, T. C.; Bliss, F. A.; Hall, T. C. Bean lectins. Part I: Relationships between agglutinating activity and electrophoretic variation in the lectin containing G2/albumin seed proteins of French bean (*Phaseolus vulgaris* L.). *Theor. Appl. Genet.* **1982**, *62*, 263−271.

(9) Leavitt, R. D.; Felsted, R. L.; Bachur, N. R. Biological and biochemical properties of *Phaseolus vulgaris* isolectins. *J. Biol. Chem.* **1977**, *252*, 2961−2966.

(10) D'Costa, S. S.; Hurwitz, J. L. Phytohemagglutinin inhibits lymphoid tumor growth in vitro and in vivo. *Leuk. Lymphoma.* **2003**, *44*, 1785−1791.

(11) Ye, X. Y.; Ng, T. B.; Tsang, P. W. K.; Wang, J. Isolation of a homodimeric lectin with antifungal and antiviral activities from red kidney bean (*Phaseolus vulgaris*) seeds. *J. Protein Chem.* **2001**, *20*, 367−375.

(12) Stefani, S. S.; Moore, C. D. Effect of phytohemagglutinin on skin allograft survival in mice. *J. Immunol.* **1970**, *104*, 780−784.

(13) Calderon, R. A.; Cordoba, F. Immunosuppressive activity of *Phaseolus coccineus* and *Phaseolus vulgaris* extracts in mice. *Eur. J. Immunol.* **1976**, *6*, 522−555.

(14) Gonzalez de Mejia, E.; Grajeda-Cota, P.; Celada, E.; Valencia, M. E. Characterization of the nutritional potential of tepary bean (*Phaseolus acutifolius*) grown in Mexico. *Arch Latinoam Nutr.* **1988**, *38*, 907−924.

(15) De Mejia, E. G.; Del Carmen Valadez-Vega, M.; Reynoso-Camacho, R.; Loarca-Pina, G. Tannins, trypsin inhibitors and lectin cytotoxicity in tepary (*Phaseolus acutifolius*) and common (*Phaseolus vulgaris*) beans. *Plant Foods Hum. Nutr.* **2005**, *60*, 137−145.

(16) Pratt, R. C.; Singh, N. K.; Shade, R. E.; Murdock, L. L.; Bressan, R. A. Isolation and partial characterization of a seed lectin from tepary bean that delays bruchid beetle development. *Plant Physiol.* **1990**, *93*, 1453−1459.

(17) Vargas-Albores, F.; De la Fuente, G.; Agundis, C.; Cordoba, F. Purification and characterization of a lectin from *Phaseolus acutifolius* var. latifolius. *Prep. Biochem.* **1987**, *17*, 379−396.

(18) Reynoso-Camacho, R.; Gonzalez de Mejia, E.; Loarca-Pina, G. Purification and acute toxicity of a lectin extracted from tepary bean (*Phaseolus acutifolius*). *Food Chem. Toxicol.* **2003**, *41*, 21−27.

(19) Ochoa, J. L.; Kristiansen, T. Purification and partial characterization of an agglutinin from *Phaseolus coccineus var. alubia*. *Biochim. Biophys. Acta.* **1982**, *705*, 396−402.

(20) Bradford, M. A rapid and sensitive method for the quantitation of protein utilizing the principles of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(21) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350−356.

(22) Bidlingmeyer, B. A.; Cohen, S. A.; Tarvin, T. L. Rapid analysis of amino acids using pre-column derivatization. *J. Chromatogr.* **1984**, *33*, 93−104.

(23) Zanetta, J. P.; Timmerman, P.; Leroy, Y. Gas-liquid chromatography of the heptafluorobutirate derivatives of O-methyl-glycosides on capillary columns: a method for the quantitative determination of monosaccharide composition of glycoproteins and glycolipids. *Glycobiology* **1999**, *9*, 255−266.

(24) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(25) Zenteno, E.; Ortega, M.; Qin, Z.; Montreuil, J.; Debray, H. Fast purification of *Phaseolus vulgaris* isolectins. *Prep. Biochem.* **1994**, *24*, 175−183.

(26) Shevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. *Anal. Chem.* **1996**, *68*, 850−858.

(27) Lyons, A. B.; Parish, C. R. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods.* **1994**, *171*, 131−137.

(28) Spiro, R. G.; Bhoyroo, V. D. Structure of the O-glycosidically linked carbohydrate units of fetuin. *J. Biol. Chem.* **1974**, *249*, 704−711.

(29) Spik, G.; Bayard, B.; Fournet, B.; Strecker, G.; Bouquelet, S.; Montreuil, J. Studies on glycoconjugates. LXIV. Complete structure of two carbohydrate units of human serotransferin. *FEBS Lett.* **1975**, *50*, 296−304.

(30) Sharon, N.; Lis, H. The structural basis for carbohydrate recognition by lectins. *Adv. Exp. Med. Biol.* **2001**, *491*, 1−16.

(31) Loris, R.; Hamelryck, T.; Bouckaert, J.; Wyns, L. Legume lectin structure. *Biochim. Biophys. Acta* **1998**, *1383*, 9−36.

(32) Zenteno, E.; Vázquez, L.; Chávez, R.; Córdoba, Wieruszeski, J.; Montreuil, J.; Debray, H. Specificity of the isolectins from the cactus plant *Machaerocereus eruca* for oligosaccharides from mucin. *Glycoconjugate J.* **1995**, *12*, 699−706.

(33) Rutella, S.; Rumi, C.; Lucia, M. B.; Barberi, T.; Puggioni, P. L.; Lai, M.; Romano, A.; Cauda, R.; Leone, G. Induction of CD69 antigen on normal CD4+ and CD8+ lymphocyte subsets and its relationship with the phenotype of responding T-cells. *Cytometry* **1999**, *38*, 95−101.

(34) Kaneda, Y.; Whittier, R. F.; Yamanaka, H.; Carredano, E.; Gotoh, M.; Sota, H.; Hasegawa, Y.; Shinohara, Y. The high specificities of *Phaseolus vulgaris* erythro- and leukoagglutinating lectins for bisecting GlcNAc or beta 1−6-linked branch structures, respectively, are attributable to loop B. *J. Biol. Chem.* **2002**, *277*, 16928−16935.

(35) Hammarstrom, S.; Hammarstrom, M. L.; Sunblad, G.; Arnarp, J.; Longren, J. Mitogenic leukoagglutinin from *Phaseolus vulgaris* binds to a pentasaccharide unit N-acetyllactosamine-type glycoprotein glycans. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 611−615.

(36) Yamashita, K.; Hitoi, A.; Kobata, A. Structural determinants of *Phaseolus vulgaris* erythroagglutinating lectin for oligosaccharides. *J. Biol. Chem.* **1983**, *258*, 14753−14755.

(37) Green, E. D.; Baenziger, J. U. Oligosaccharide specificities of *Phaseolus vulgaris* leukoagglutinin and erythro-agglutinin phytohemagglutinins. Interactions with N-glycanase-released oligosaccharides. *J. Biol. Chem.* **1987**, *262*, 12018−12029.

(38) Pérez-Campos, E.; Sierra, C.; Lascurain, R.; Espinoza, B.; Bouquelet, S.; Debray, H.; Zenteno, E. The erythroagglutinin from *Phaseolus coccineus* var. alubia: Chemical characterization, sugar specificity and effect on coagulation factors. *J. Agric. Food Chem.* **1997**, *45*, 3747−3752.

(39) Cumming, D. A.; Hellerqvist, C. G.; Harris-Brandts, M.; Michnick, S. W.; Carver, J. P.; Bendiak, B. Structures of asparagine-linked oligossaccharides of the glycoprotein fetuin having sialic acid linked to *N*-acetyl-D-glucosamine. *Biochemistry* **1989**, *28*, 6500−6512.

(40) Pierce-Cretel, A.; Decottignies, J. P.; Wieruszeski, J. M.; Strecker, G.; Montreuil, J.; Spik, G. Primary structure of twenty three neutral and monosialylated oligosaccharides O-glycosidically linked to the human secretory immunoglobulin A hinge region determined by a combination of permethylation analysis and 400-MHz 1H-NMR spectroscopy. *Eur. J. Biochem.* **1989**, *182*, 457−476.

(41) Fournet, B.; Montreuil, J.; Strecker, G.; Dorland, L.; Haverkamp, J.; Vliegenthart, J. F. G.; Binette, J. P.; Schmid, K. Determination of the primary structures of 16 asialo-carbohydrate units derived from human plasma α_1-acid glycoprotein by 360 ^{1}H NMR spectroscopy and permethylation analysis. *Biochemistry* **1978**, *17*, 5206−5214.

(42) Montreuil, J. Spatial structures of glycan chains in relation to metabolism and function. *Pure Appl. Chem.* **1984**, *56*, 859−877.

(43) Hakomori, S. Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism. *Cancer Res.* **1996**, *56*, 5309−5318.

(44) Asada, M.; Furukawa, K.; Segawa, K.; Endo, T.; Kobata, A. Increased expression of highly branched N-glycans at cell surface is correlated with the malignant phenotypes of mouse tumor cells. *Cancer Res.* **1997**, *15*, 1073−1080.

(45) Yamamoto, H.; Swoger, J.; Greene, S.; Saito, T.; Hurh, J.; Sweeley, C.; Leestma, J.; Mkrdichian, E.; Cerullo, L.; Nishikawa, A.; Ihara, Y.; Taniguchi, N.; Moskal, J. R. β-1,6-N-acetylglucosamine-bearing N-glycans in human gliomas: implications for a role in regulating invasivity. *Cancer Res.* **2000**, *60*, 134−142.

(46) Castillo-Villanueva, A.; Abdullaev, F. Plant lectins and their effects on cancer. *Rev. Invest. Clin.* **2005**, *57*, 55−64.

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