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A COMPARATIVE STUDY ON THE PURIFICATION OF THE *AMARANTHUS LEUCOCARPUS SYN. HYPOCONDRIACUS* LECTIN

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ABSTRACT

Amaranthus leucocarpus lectin is a homodimeric glycoprotein of 35 kDa per sub-unit, which interacts specifically with N-acetyl-galactosamine. In this work, we compared different glycoproteins that contain Galβ1-3 Ga1NAcα1-3 Ser/Thr or Ga1NAcα1-3 Ser/Thr in their structure as ligands to purify the *A. leucocarpus* lectin. From the glycoproteins tested, fetuin was the most potent inhibitor of the hemagglutinating activity and the better ligand for lectin purification; however, the use of desialylated stroma from erythrocytes represented the cheapest method to purify this lectin. O-linked glycans released from the glycoproteins used as affinity matrix and those from different erythrocytes were less inhibitory than parental glycoproteins. The NH_2-terminal of the lectin is blocked; moreover, this is the only example of a lectin isolated from this genus to be a glycoprotein. Analysis of the glycoprotein sequences with inhibitory activity for the lectin, showed a different pattern in the O-glycosylation, which confirms that *A. leucocarpus* lectin recognizes conformation and, probably, distances among O-linked glycans moieties.

INTRODUCTION

Lectins are carbohydrate binding proteins or glycoproteins of non-immune origin, and with no enzymatic activity.[1] Due to their specific binding properties, they have been used in studies of cell surface architecture, blood group typing, isolation, and characterization of oligosaccharide structures.[2] Lectins are responsible for cell surface recognition in bacteria, animals and plants[3] Plant lectins are widely used in immunology, cell biology, and cancer research.[4]

Lectins from different species from the *Amaranthaceae* group have been identified.[5,6] *Amaranthus leucocarpus* syn *hypocondriacus* is a Mexican representative of the *Amaranthaceae* family, which possesses high nutritional value due to its protein content and the considerable proportion of essential amino acids.[7] *A. leucocarpus* possesses a lectin specific for GalNAc (N-acetyl-galactosamine).[8] The lectin from *A. leucocarpus* (ALL) induces immunosuppression in animals,[9] recognizes unstimulated murine peritoneal macrophages, and recognizes $CD4^+$ mouse medullary thymocytes sub-population[10] as well. This lectin has been recently demonstrated to interact with O-glycosyl proteins in some neurodegenerative process.[11] ALL agglutinates preferentially human erythrocytes with the M phenotype,[12] suggesting that the density or the topographical presentation of saccharidic moieties in the lectin receptor plays a relevant role in the interaction with ALL. In this work, we compared different glycoproteins that share

Galβ1-3 GalNAcα1-OSer/Thr (T-antigen) and Ga1NAcα1-0Ser/Thr (Tn-antigen) in their structure as specific chromatography ligands and used them to improve the method for the purification of ALL. We also describe the chemical characteristics of this lectin.

EXPERIMENTAL

Reagents

Chromatographic materials, Sephadex G-25 and Sepharose 4B, were from Pharmacia Biotechnology (Uppsala, Sweden). Fetuin, human immunoglobulin A (IgA), ovine and bovine submaxillary gland mucin, cyanogen bromide, as well as sugars, were from Sigma (Sigma Fine Chem. Co., St. Louis, MO, USA). All other reagents were of analytical grade. Glycans from human IgA were a kind gift of Prof. A. Pierce, from the Université des Sciences et Techniques de Lille, France.

Lectin Extraction

Amaranthus leucocarpus seeds were obtained in Tulyehualco (Mexico). The seeds were ground to a fine powder in a grinder and the seed meal was deslipidated with petroleum ether. Their soluble proteins were extracted by agitation overnight with 10 volumes of 0.15 M sodium chloride (SSI) at 4°C. The pH of the extract was adjusted to 4 with 1M acetic acid and the suspension allowed to stand overnight at 4°C. The clear supernatant (crude extract), obtained by centrifugation at 3000 x g for 10 minutes, was stored at 4°C for further studies.

Lectin Purification

Purification by stroma column

Erythrocyte membranes (stroma) were obtained by lysis of human red blood cells group O^M according to Dodge *et al.*[12] The membranes were fixed with 1% glutaraldehyde overnight at 4°C, then the stroma were washed with distilled water and freeze dried. Two hundred milligrams of erythrocyte stroma was physically entrapped in a chromatographic column with Sephadex G-25 as described.[13] The column was equilibrated with SSI (0.15 M NaCl). The crude extract (160 mg of protein) was applied to the stroma column (25 x 1.2 cm), and the unretained material was eluted with SSI at a flow rate of 15 mL/h, until the A_{280} of the collected fractions was below 0.01. The bound lectin was eluted with acetic acid (3%) and the pH of each collected fraction was adjusted to 6

with 1N NaOH and tested for protein concentration and hemagglutinating activity. In control assays, we eluted the lectin from the affinity chromatography column by adding 200 mM GalNAC, and results indicate that, in both eluting systems, the column yields the same lectin concentration.

Purification with immobilized glycoproteins

140 mg of each glycoprotein (IgA, fetuin, bovine, or ovine submaxillary mucin) was coupled to 20 mL of CNBr-activated Sepharose 4B CNBr.[14] The columns were equilibrated with SSI at a flow rate of 15 mL/h. The *A. leucocarpus* crude extract (160 mg of protein) was applied to each column and lectin elution was performed as described above.

Hemagglutinating

Erythrocytes from different animal species were obtained from the animal facilities at the School of Medicine, UNAM. Updated human erythrocytes from healthy donors were obtained from the Central Blood Bank, IMSS, Mexico. Hemagglutinating activity was assayed in microtiter U plates (NUNC, Denmark) by the two-fold serial dilution procedure.[15] The agglutinating activity was tested with either 2% (w/v) untreated erythrocyte suspension in phosphate buffered saline (PBS: 0.01 M sodium phosphate, 0.14 M sodium chloride, pH 7.2) or with neuraminidase-treated (0.1 U per 0.5 mL of packed erythrocytes at 37°C for 30 min) or pronase-treated (100 μg per 0.5 mL of packed erythrocytes at 37°C for 30 min) erythrocytes. The hemagglutinating titer is reported as the inverse of the last dilution with agglutinating activity.

Preparation of Glycans

Desialylated stromata from donkey, rabbit, and human O^M erythrocytes were obtained by the method described by Dodge; 12 lipids were eliminated by three sequential extractions with chloroform:methanol (2:1, v/v) followed by chloroform:methanol (1:2, v/v). The glycoproteins thus obtained were dried under nitrogen.[18] Glycans were obtained by mild alkaline treatment, incubating 100 mg of each glycoprotein in O.1M NaOH and 1M $NaBH_4$ at 45°C, 36 h; then, adding Dowex 50X8-H+ to stop the reaction. The fractions were desalted on a column containing Bio-Gel P2 (60 x 2 cm) equilibrated with water and freeze-dried. Glycans and N-glycopeptides were separated by filtration on an Ultrogel ACA 202 column (100 x 2.6 cm), equilibrated in 0.01 M Tris/HCI, 0.17 M NaCl, pH 7.4. Stromata from erythrocytes and glycoproteins and glycans were desialylated by incubation at 100°C for 1 h in the presence of 0.02 N sulfuric acid, as described by Spiro and Bhoyroo,[16] desalted on a Biogel P-2 column (2 x

60 cm), equilibrated with 0.5 M acetic acid, and lyophilized until use. Asialo-fetuin (2 mM) was then treated with 1 U of β-galactosidase from jack bean at 37°C, 24 h, as described in ref.[17], then asialo-agalacto-fetuin was desalted on a Biogel P2 column equilibrated with distilled water and freeze dried. Glycan from asialofetuin was obtained by digestion with 1 mU of endo-N-acetylgalacosaminidase from *Diplococus pneumoniae* as described.[8] The carbohydrate composition of each glycan and modified glycoproteins was determined by gas-liquid chromatography (see below).

Sugar Specificity

The sugar specificity of the lectin was determined by comparing the inhibitory activity of sugars, glycoproteins, their desialylated derivatives, or glycans on the hemagglutination induced by the lectin against pronase-treated human erythrocytes. Results are expressed as the minimal concentration required to completely inhibit four hemagglutinating doses.

The molar concentration of the glycoproteins was determined according to their molecular weight. In desialylated glycoproteins, we subtracted the number of sialic acid molecules released from the native protein and the molar concentration of glycans was calculated on the basis of their monosaccharide content as determined by gas-liquid chromatography.

Prediction of O-Glycosylation Sequences

NetOglyc program[18] was used for the prediction of fetuin, IgA, bovine, and ovine submaxillary mucin O-glycosylation sequences.

Analytical Methods

Protein concentration was determined by the method of Bradford[19] with Coomassie blue R-250, using bovine serum albumin as standard. Carbohydrate concentration was determined by the method of Dubois,[20] using lactose as standard. Carbohydrate composition analysis was carried out by methanolysis in the presence of meso-inositol (Sigma Chem., St. Louis, MO) as internal standard; the per-O-trimethyl silylated methyl glycosides (after N-re-acetylation) were analyzed by gas chromatography using a capillary column (25 x 0.32 mm) of 5% Silicone OV 210, (Applied Science Lab., Buffalo, NY), in a Varian 2100 gas chromatograph (Orsay, France) equipped with a flame detector and a glass solid injector; the carrier gas was helium at a pressure of 0.6 bar, and the oven temperature was programmed

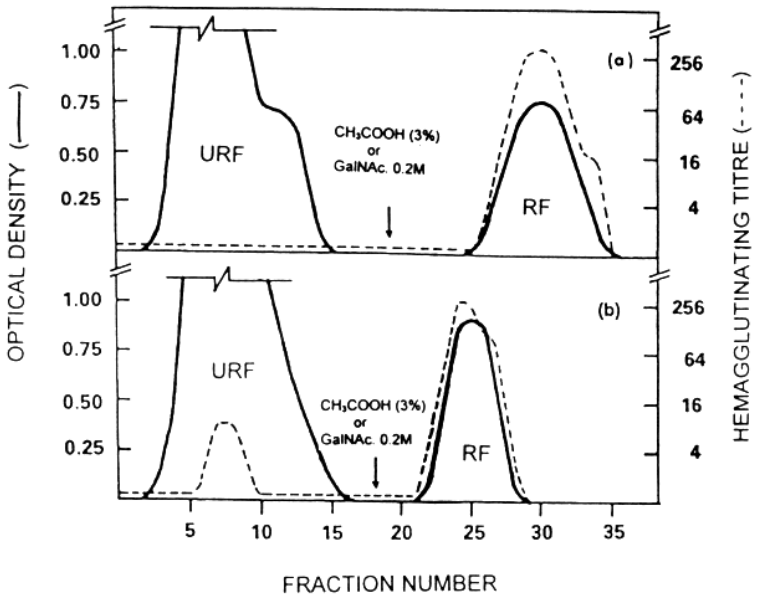

Figure 1. Purification of *A. leucocarpus* lectin. 160 mg of crude extract was applied onto a column containing A) Stroma from human erythrocytes or B) IgA-Sepharose 413. The unretained material was eluted with 0.15 M NaCl, and the lectin was eluted with 3% acetic acid or 200mM of GalNAc. The pH of each fraction was neutralized with NaOH and the OD at 280 nm and hemagglutinating activity in presence of human erythrocytes type O was tested. A similar elution pattern to that of the stroma was obtained with the columns prepared with fetuin, ovine, and bovine submaxillary mucins. In the same conditions, the column containing IgA-Sepharose 4B is saturated, and hemagglutinating activity is detected in the non-retained fraction.

from 150 °C to 250 °C at 3 °C per min as described by Zanetta et al.[21] Polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a slab gel apparatus according to the method of Laemmli.[22] Coomassie Brilliant Blue R-250 was used for staining.

Amino Acid Analysis

A 100 µg sample was hydrolyzed under vacuum with 2 mL of 6M HCl at 110 °C in sealed tubes for 24, 48, and 72 h. The samples were analyzed on an automatic amino acid analyzer Durrum 500, according to Bidlingmeyer *et al* .[23] using Nor-leucine as internal standard.

Table 1

Purification Process of *A. leucocarpus*[a] Lectin by Different Affinity Chromatography Matrices

Fraction	Protein (mg)	HAU[b]	Specific Activity[c]	Protein Yield (%)
Crude Extract	812	44800	55.2	100
Fetuin	42	38100	907.1	5.1
Stroma	40	32800	820	4.9
Ovine Mucin	35	32100	917.1	4.3
Bovine Mucin	31	30000	967.7	3.8
IgA	28	27900	996.4	3.4

[a]From 10 g seed meal. [b] Hemagglutinating units with human erythrocytes type O. [c]HAU/mg protein.

Amino acid sequences were obtained from SDS-PAGE-separated ALL. After electroblotting on a PVDF membrane, the band containing ALL was excised from the blot and sequenced on a Beckman Instrument Inc (Fullerton, CA, USA) Model LF3000 protein sequencer.

RESULTS

Lectin purification

From the *Amaranthus leucocarpus* seed extract, we purified in a single chromatography step, the lectin, which represents, in general, 8% of the total soluble protein. The different affinity chromatography supports, i.e., fetuin, ovine and bovine submaxillary mucin, and stroma from erythrocytes, are powerful ligands for its purification. Human IgA showed lower capacity to retain the lectin (Table 1 and Fig. 1). The unretained material was always eluted with SSI and the lectin was eluted from the affinity matrix with 3% acetic acid (Fig. 1), a similar elution pattern was obtained when we added O.1M GalNAc in SSI to each column.

Although the yield of purified lectin varies according to the affinity matrix used, we consider that the optimal purification system corresponds to fetuin and erythrocyte stroma affinity columns. Our results indicate that the purified lectin corresponds to 5% of protein of the crude extract. With any of the chromatography supports used, more than 60% of hemagglutinating activity was recovered and the

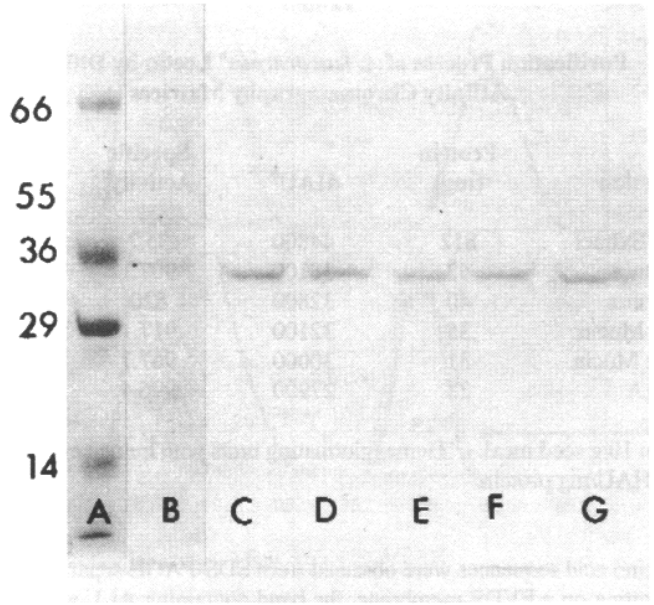

Figure 2. SDS-polyacrylamide electrophoresis of the purified *Amaranthus leucocarpus* lectin: C-G) Purified lectin 5 μg from human stroma, fetuin, ovine, and bovine submaxillary gland mucins and IgA-Sepharose 4B columns, respectively. b) 25 μg of *A. leucocarpus* crude extract. a) Molecular weight markers: bovine serum albumin (66 kDa); ovalbumin (45 kDa); glyceraldehyde 3 phosphate deshydrogenase, rabbit muscle (36 kDa), carbonic anhydrase (29 kDa), and α-lactoglobulin from bovine milk (14 kDa).

specific activity was increased when compared with the crude extract. The purification process of the lectin is summarized in Table 1. As shown in figure 2, the purified fractions obtained from any of the affinity systems used are homogeneous, showing a single band in SDS-PAGE.

Lectin Characterization

ALL is a glycoprotein of 35 kDa, as revealed by SDS-PAGE (Fig. 2). As previously indicated,[13] the lectin contains 52.8% of hydrophobic, 15.2% of polar, and 31% of charged amino acids, and three cystein residues per sub-unit. ALL is a glycoprotein with a sugar content of 8%. Monosaccharide analysis indicates the glycans to be of the N-glycosidic type with galactose, mannose, N-acetyl-glucosamine, and xylose, in the molar ratio of 4:3:5:1. The NH_2-terminal residue of ALL is blocked.

Table 2

Hemagglutinating Activity of the *Amaranthus leucocarpus* Lectin[a]

Erythrocytes	HA Titer Native	Nanase	Pronase
Donkey	1024	8096	8096
Rabbit	256	1024	2056
Mouse (CD-1)	16	64	64
Rat	16	32	32
Pig	32	256	256
Sheep	16	128	128
Human O^M	64	1024	512
Human O^N	64	256	256

[a] Lectin concentration was 16 μg/mL. Hemagglutinating titer is reported as the inverse of the last dilution with agglutinating activity tested in the presence of a 2% solution of native neuraminidase, or pronase treated erythrocytes.

Hemagglutinating Activity

ALL showed hemagglutinating activity towards erythrocytes from different animal species; erythrocytes from donkey are better recognized than cells from other species. Treatment of erythrocytes with neuraminidase or pronase increased the hemagglutinating capacity of the lectin. It is interesting to note that human erythrocytes are agglutinated independently from the ABO blood group system; however, the hemagglutinating titer of the lectin is higher in the presence of erythrocytes presenting the M blood phenotype treated with neuraminidase or pronase than with erythrocytes containing N phenotype. The presence of N or M phenotype in pronase treated cells was confirmed using a specific commercial antiserum.

The lectin from ALL shows hemagglutinating activity in the presence of all erythrocytes tested, although the erythrocytes from donkey and rabbit were sixty-four times more agglutinated than mouse erythrocytes (Table 2). Hemagglutinating activity increased after treatment of erythrocytes with neuraminidase or pronase; however, as previously described,[8] there is a significant difference in the increase of hemagglutinating titer in the presence of human erythrocytes with the M phenotype as compared with the erythrocytes with the N phenotype. No differences were observed among human erythrocytes in the ABO blood group system.

Table 3

Inhibition of the Hemagglutinating Activity of *A. leucocarpus* Lectin with Glycoproteins and Glycans[a]

Compound	Concentration (mM)
GalNAc	62
Human IgA	0.004
Human Asialo IgA	0.001
Fetuin	0.0005
Asialo-Fetuin	0.0001
Agalacto-Fetuin	0.0001
Bovine Mucin	0.05
Bovine Asialomucin	0.01
Ovine Mucin	0.01
Ovine Asialomucin	0.005
Asialo Glycan from Human Erythrocyte[b]	15
Donkey Erythrocyte	15
Rabbit Erythrocyte	15
Fetuin	15
Fetuin[c]	1
IgA	15

[a] Minimum concentration of glycoprotein or glycans to inhibit 4 hemagglutinating units of *A. leucocarpus* lectin (Titer = 4). Tests were performed in the presence of desialylated human erythrocytes O^{M}. [b] O-glycosidically linked glycans were liberated from desialylated glycoproteins with reductive β-elimination and contains Gal/GalNAc-ol.
[c] O-glycosidically linked glycans liberated from asialo-fetuin by treatment with endo-N-acetylgalactosaminidase treatment contains Gal/GalNAc.

Sugar Specificity

From all the monosaccharides tested, only GalNAC inhibited the hemagglutinating activity of *A. leucocarpus* lectin and glycoproteins containing GalNAc are powerful inhibitors. From the glycoproteins tested, the most potent inhibitor was fetuin; and to a lesser extent, IgA, ovine, and bovine submaxillary were also more powerful inhibitors than GalNAc. The glycans released from these glycoproteins by reductive β-elimination or by enzymatic treatment had inhibitory capacity, but they were four and sixty times, respectively, more powerful inhibitors than GalNAc, but less inhibitory than parental O-glycosylproteins (Table 3). All

Table 4

Prediction of O-Glycosylable Sequences from Glycoproteins[a]

Glycoprotein	Sequence
Ovine Mucin	S T* T* G S T* S
Bovine Mucine	S* E S T* T* Q L P
Fetuin	A G P T* S A A G
	V A S* V V V**
	G P T* P S A**
	A P S*A V P**
IgA	T*P S T*P S T*P
Glycophorin A^M	S* S* T* T E V A M
Glycophorin A^N	L S* T* T* G V A M

[a] * indicates the putative glycosylation site, based on netOglyc program[18] and ** proposed by Smith, et al.[36]

the glycans liberated from desialylated fetuin, IgA, and stromata from donkey, rabbit, and human O^M erythrocytes contained Gal and GalNAc-ol, and inhibited the hemagglutinating activity of *A. leucocarpus* lectin at the same concentration (Table 3).

Prediction of O-Glycosylation Sequences

Results from the prediction of sequences most susceptible to O-glycosylation in fetuin, IgA, ovine, and bovine submaxillary mucin and glycophorin A^N and A^M are summarized in Table 4. O-glycosylable sequences in IgA, ovine and bovine submaxyllary mucin are rich in serine and threonine residues, indicating that they possess different putative O-glycosylation sites; whereas, the O-glycosylable sequence in fetuin has only one residue able to link O-glycans.

DISCUSSION

Plant lectins represent a group of well-preserved proteins in evolution. This property is observed even in their sugar-binding specificity. 24 Lectins from Amaranteceae family have been demonstrated in *A. caudatus*,[5] *A. cruentus*,[6] and *A. leucocarpus*.[13] These lectins represent important tools to characterize O-glycosidically linked glycans,[25] and some authors have suggested their capacity to specifically recognize sugar structures in tissues and tumors.[9-11,25] In this work, we compared the use of the stroma of human erythrocytes type O^M, IgA, ovine and bovine submaxillary gland mucins, and fetuin as affinity chromatography ligands to find a rapid and economic method to purify *A. leucocarpus* lectin. We obtained, with all these purification systems, an increase in the specific activity as compared with the crude extract. Our results indicate that, if all these ligands are adequate to purify *A. leucocarpus* lectin, the most economic method to purify the lectin was the stroma column prepared from updated erythrocytes. This process can also be used to purify other lectins with specificity for GalNAc or Galβ1,3 GalNAcαcal-3Ser/Thr.[26]

In general, the lectins from the Amarantaceae group are homodimeric proteins with a range of 33 (*A. caudatus* and *A. cruentus*)[5,6] to 35 kDa per subunit (*A. leucocarpus*). The difference in the molecular weight shown by *A. leucocarpus* seems to correspond to a glycan moiety, since this is the only lectin from this group to be glycosylated. In the amino acid sequences of all the lectins from this group, the amino terminal is blocked and *A. leucocarpus*, as we show in this work, is not the exception.

The ability of lectins to bind carbohydrates and their capacity to detect subtle variations in the conformation of carbohydrate structures found in glycoconjugates of the cell surface depends on their 3-D structures.[27-29] *A. leucocarpus* lectin recognizes O-glycosidically-linked glycans and glycoproteins containing these structures. However, the tested glycoproteins, fetuin, IgA, bovine and ovine submaxillary mucins, showed different capacities to inhibit the hemagglutinating activity of the lectin. Fetuin and its asialo-form were the most potent inhibitors; elimination of galactose residues does not modify, significantly, the interaction with the lectin, indicating that *A. leucocarpus* lectin reacts strongly with T (Galβ1,3GalNAcαl,0Ser/Thr) and Tn (GalN Acα1,0Ser/Thr) structures.

This result is confirmed by the specific interaction with ovine submaxillary gland mucin and its asialo-form, since this glycoprotein contains only NeuAcα2,3GalNAc Ser/Thr.[30] Fetuin possesses three O-glycosidically-linked glycans and thirteen N-glycosidically linked glycans.[16] IgA and bovine submaxillary gland mucin also contain O-glycans.[30,31] The analysis of O-glycosylable sequences in these glycoproteins indicated that in contrast to fetuin,

ovine, bovine submaxillary gland mucins, and IgA these sites are not organized in clusters; hence, it is possible that lectin interaction requires a specific distance among the O-glycosylable sites.

This suggestion is supported by the fact that O-linked glycans from any of the glycoproteins tested, released by reductive β-elimination (containing Galβ1,3GalNAc-ol) or by enzymatic treatment (Galβ1,3GalNAc), including those from all types of asialo-erythrocyte stromata, inhibit to the same extent the activity of the lectin. It is noteworthy that liberated glycans present a drastically reduced inhibitory effect, when compared to the parental glycoproteins, being only four and sixty times more powerful than GalNAc.

ALL agglutinates, preferentially, the human erythrocytes with the M phenotype. The M and N phenotypes in human erythrocytes are present in glycophorins A^M and A^N and both glycoproteins possess the same rate of O-glycosylation (see Table 4) although they show differences at their NH_2-terminal, glycophorin A^M possessing a serine residue and A^N leucine residue.[32] Moreover, our results strongly suggest that ALL recognizes conformation and distances between O-glycosidically-linked GalNAC.

O-glycosidically linked glycans attached to membrane and cell surfaces (conforming mucin and mucin-like structures) play important roles in modulating the immune response, inflammation, and tumorigenesis.[33,34] These structures are responsible for the 3-D organization of glycoproteins.[35] Based on the aforementioned data, our results strongly suggest that the lectins from the Amaranthus genus, particularly *A. leucocarpus*, represent a tool for the study of mucin structures and their biological role.

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