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Specificity of *Amaranthus leucocarpus* lectin

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We have demonstrated that *Amaranthus leucocarpus* lectin hemagglutinating activity was powerfully inhibited by the T-antigen, containing Gal(β1-3)GalNAc(α1-3)Ser/Thr, and the T_n-antigen, which contains GalNAc(α1-3)Ser/Thr. This suggests that the acetamido group at C-2 and the axial –OH at C-4 of the *N*-acetyl-D-galactopyranosylamine ring are important for lectin binding. The hemagglutination assays also established that desialylated and Pronase-treated human type *O* erythrocytes with an M phenotype were better recognized than erythrocytes from all other blood groups. The recognition was dependent on pH and ionic strength.

Keywords: plant lectin, *Amaranthus leucocarpus*, T and T_n antigen-specific lectin

Amaranthus leucocarpus is a Mexican species of the *Amaranthus* genus with a high nutritional value due to its protein content and the considerable proportion of essential amino acids it possesses [1]. Previous reports have shown similarities between the lectins isolated from *A. caudatus* [2, 3], *A. cruentus* [4, 5] and *A. leucocarpus* [6], such as two 33,000–36,000 Da monomer units forming a native 66,000 Da homodimer, presence of common epitopes and inhibition of their hemagglutinating activity by *N*-acetyl-D-galactosamine and fetuin, a glycoprotein containing both O- and N-glycosidically linked glycans [7]. Similarly to *A. leucocarpus*, the Gal(β1-3)GalNAc specificity of *A. caudatus* lectin has only recently been determined [3]; however, *A. leucocarpus* induces immunosuppression in animals and is mitogenic for murine spleen lymphocytes [6, 8] where *A. caudatus* does not exert any such effect [2].

In order to identify the nature of these discrepancies, we tried to establish (a) the fine sugar specificity of the *A. leucocarpus* lectin, and (b) whether human erythrocytes with phenotypes in which *N*-acetylgalactosamine residues represent important determinants were specifically agglutinated by the lectin.

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Materials and methods

Materials

Amaranthus leucocarpus seeds obtained in Tulyehualco (Mexico) were identified at the Centro de Investigaciones Biológicas, UAEM, Mexico. The lectin was purified by affinity chromatography on a column containing human type O red blood cell stroma as already described [6]. Ultrogel ACA-202 was from IBF-Biotechnics (Clichy, France); Bio-Gel P-4 and Bio-Gel P-2 were from Bio-Rad (Vitry sur Seine, France). Mucine from bovine submaxillary gland, mucin grade II from porcine stomach, fetuin from fetal calf serum, human α_1-acid glycoprotein, Pronase (*Streptomyces griseus*, Sigma fraction XXV), neuraminidase (*Vibrio cholerae*, Sigma fraction V, EC 3.2.1.18) as well as other sugars, chemicals and proteins were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Human serum IgA as well as its O-glycans that contain Gal(β1-3)GalNAc [9] were gifts from Professor G. Spik; α(2-3)- and α(2-6)-sialyllactose as well as ovine submaxillary mucin were a gift from Dr. G. Strecker (Université des Sciences et Techniques de Lille Flandres-Artois, France).

Analytical methods

Protein concentration was determined by the method of Lowry *et al.* [10] using bovine serum albumin as a standard.

Total carbohydrate was determined by the phenol–sulfuric method [11] using lactose as standard. Carbohydrate composition analysis was carried out by methanolysis in the presence of *meso*-inositol as internal standard and the per-*O*-trimethyl silylated methyl glycosides (after N-reacetylation) were analysed by gas–liquid chromatography in a capillary column (25 mm × 0.32 mm) of silicone OV 101 [12].

Preparation of glycans and glycopeptides

Fetuin glycopeptides were obtained by Pronase digestion (1 mg of enzyme per 10 mg of glycoprotein were incubated for 48 h at 37 °C). The O- and N-glycosyl peptides were fractionated by gel-filtration on a column (1.6 cm × 100 cm) containing Bio-Gel P-4 equilibrated with 0.05 M pyridine acetate, pH 4.5 [13]. O-Glycosidically-linked glycans from fetuin were liberated by alkaline reductive treatment [14], and further fractionated by gel filtration on a column of Ultrogel ACA-202 (1.6 cm × 100 cm) equilibrated in 0.01 M Tris/HCl, pH 7.4, 0.17 M NaCl, 0.02% NaN_3; the O-glycans and the N-glycosyl peptides were desalted on Bio-Gel P-2 equilibrated with water and kept lyophilized until use [15].

Glycoproteins, glycopeptides or 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], and desalted on a Bio-Gel P-2 column (2 cm × 60 cm) equilibrated with water.

Endo-*N*-Acetylgalactosaminidase digestion of asialofetuin was carried out at 37 °C for 48 h with 1 mU of *Diplococcus pneumoniae* endo-*N*-acetylgalactosaminidase (EC 3.2.1.97, Boehringer, Mannheim, Germany). The released O-glycosidically linked glycans were purified by descending paper chromatography for 16 h on Whatman No. 3 paper in ethyl acetate–pyridine–acetic acid–water, 5:5:1:3 by vol. [17]. O-Glycosidically linked glycans from porcine stomach mucin were obtained by alkaline-borohydride degradation [18] and fractionated by high performance liquid chromatography on an ODS-column (Alltman, USA) as described earlier [15]. Structural characterization of the obtained oligosaccharide-alditols was completed by methylation analysis and by 500-MHz ^{1}H-NMR studies.

Hemagglutinating activity

Human erythrocytes type A,B,O,M,N,MN,Le^a, Le^b, P, S and Kell from healthy human donors were from the Central Blood Bank, IMSS, Mexico. *Amaranthus leucocarpus* lectin hemagglutinating activity was assayed in microtiter U plates (NUNC, Denmark) according to a twofold serial dilution procedure [19]. The hemagglutinating activity was tested with either 2% (w/v) untreated erythrocyte suspension in phosphate buffered saline (0.01 M sodium phosphate, 0.14 M sodium chloride, pH 7.2; PBS) or with neuraminidase-treated (0.1 U per 0.5 ml of packed erythrocytes at 37 °C for 30 min), Pronase-treated (100 µg per 0.5 ml of packed erythrocytes at 37 °C for 30 min) or neuraminidase- and Pronase-treated erythrocytes. To verify that enzyme treatment did not remove the antigenic determinants on the RBC surface, we corroborated the presence of the M and N antigens after enzyme treatment with specific antibodies. The hemagglutinating titre is reported as the inverse of the last dilution with agglutinating activity.

Sugar specificity

The lectin sugar specificity was determined by comparing the inhibitory activity of various sugars, glycoproteins, or their derived glycopeptides and glycans, on the hemagglutination induced by the lectin against neuraminidase- and Pronase-treated human type O^m erythrocytes [19]. Results are expressed as the minimal concentration required to completely inhibit four hemagglutinating doses. The glycoprotein molar concentration was determined according to their molecular weight; with desialylated glycoproteins we subtracted the number of sialic acid molecules released from the native protein. The molar concentration of glycans and glycopeptides was calculated on the basis of their oligosaccharide content as determined by gas chromatography.

pH and ionic strength dependence study

Amaranthus leucocarpus lectin was exposed to a wide range (5.0–9.0) of pH before assessing its hemagglutinating activity toward neuraminidase- and Pronase-treated N and M erythrocytes. The lectin was first exhaustively dialysed against distilled water and then against a 'universal' buffer (NaCl 0.1 M; citric acid, $Na_2H_2PO_4$, barbital, glycine, 0.028 M each) the pH of which was adjusted to the desired value with 0.2 M NaOH [20].

Similarly, the effect of ionic strength on the lectin activity was assessed by exposing the lectin to different salt concentrations. The lectin was first dialysed against deionized water (conductivity = 1–2 µmoh) containing 0.2 M sucrose to prevent hemolysis, and then its agglutinating activity against 2% N or M erythrocyte suspension was tested in the presence of various concentrations of NaCl ranging from 0 to 0.2 M. Control assays were performed with isotonic saline (NaCl 0.15 M) solution alone or supplemented with 0.2 M sucrose.

Results

Blood group specificity

A. leucocarpus does not possess serological specificity for the human blood groups A, B and O [6]. Moreover, the lectin does not differentiate between Kell, Le^a, Le^b, P, M, N and S blood groups. As for A, B and O native erythrocytes, Pronase treatment increased the lectin agglutinating activity towards these cells five-fold. However, it is noteworthy that with neuraminidase-treated erythrocytes, the lectin agglutinating activity was four times more powerful with

Table 1. Hemagglutinating activity of *A. leucocarpus* lectin[a] against different human type O erythrocyte phenotypes.

Blood group	*Hemagglutinating titre of enzyme-treated or untreated erythrocytes*			
	Untreated	*Pronase*	*Neuraminidase*	*Pron./ neuram.*
Kell	2	64	64	64
Le^a	2	64	64	64
Le^b	2	64	64	64
M	2	128	256	1024
N	2	64	64	128
P	2	64	64	64
S	2	64	64	64

[a] The lectin concentration was 2.4 μg ml^{-1}. Similar results were obtained with human type A and B red blood cells with either M or N phenotype. All blood groups, with the exception of the M phenotype, possessed the N antigen.

asialo-M erythrocytes than with any other tested groups, including asialo-N red cells (Table 1). These differences were more marked when the cells were Pronase- and neuraminidase-treated. With these treated cells, only 0.002 μg of the lectin was required to induce agglutination as compared to asialo-N-erythrocytes where 0.018 μg was needed. Enzyme treatment did not remove the M and N antigens from the red blood cell surface.

Sugar specificity

To determine the carbohydrate binding specificity we conducted hapten inhibition assays of the agglutination induced by the lectin on asialo and Pronase-treated human type O^m erythrocytes. Among the tested simple sugars only *N*-acetyl-D-galactosamine inhibited the agglutination (Table 2). Addition of galactose or its derivatives galactosamine, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside and *p*-nitrophenyl-α or -β D-galactopyranoside at a concentration of 100 mM did not modify the agglutinating titre of the lectin (data not shown). The same results were obtained with lactose, α(2-3)- or α(2-6)-sialyllactose, and N-glycosylproteins, such as human α_1-acid glycoprotein, human serotransferrin and human IgG, or their derived N-glycosylpeptides.

Glycoproteins containing O-glycosidically-linked glycans such as fetuin, bovine and ovine submaxillary mucin, and human IgA were better inhibitors than *N*-acetylgalactosamine (Table 2). Interestingly enough, human milk IgA, which possesses greater amounts of complex O-glycans [21], was fourfold less inhibitory than human serum IgA. The inhibitory capacity of these glycoproteins was significantly increased after desialylation. Although O-glycosylpeptides and asialo O-glycosylpeptides were powerful inhibitors, they were less inhibitory than the parent O-glycosylproteins. Moreover, N-glycosylpeptides from fetuin, obtained by

Table 2. Carbohydrate specificity of *Amaranthus leucocarpus* lectin.[a]

Compound	*Concentration* (μM)	*Relative inhibitory potency*
N-acetylgalactosamine	62	1
Ovine submaxillary mucin	0.01	6,200
Asialo-ovine submaxillary mucin	0.005	12,400
Bovine submaxillary mucin	0.05	1,240
Asialo-bovine submaxillary mucin	0.01	6,200
Fetuin	0.0005	124,000
Asialo-fetuin	0.0001	620,000
Human milk IgA	0.004	15,500
Human serum IgA	0.001	62,000
α_1-acid glycoprotein	NI[b]	–[b]
Human lactotransferrin	NI	–
Fetuin asialo-glycan[c]	15	4.1
Fetuin asialo-glycan[d]	1	62
Fetuin asialo-*O*-glycosylpeptide	0.01	6,200
Serum IgA glycan	13	4.8
Porcine stomach mucin	NI	–
glycan I	15	4.1
glycan II	32	1.9
glycan III	NI	–

[a] Hemagglutinating activity was tested against neuraminidase- and Pronase-treated human type O red blood cells.
[b] Other sugars and glycosides not inhibitory (NI) at a 100 μM concentration included: D-galactosamine, D-galactose, D-mannose, D-glucose, L-fucose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-neuraminic acid, *p*-nitrophenyl α- or β-galactosamine, methyl α- or β-galactoside, α(2-3)- and α(2-6)-sialyllactose, lactose, *N*-acetyllactosamine, Galβ1-4(2-acetamido-2-deoxy-anhydroglucosaminitol).
[c] Fetuin asialo-glycan = Galβ1-3(acetamido-2-deoxy-1,5-anhydrogalactitol).
[d] Fetuin asialo-glycan = Galβ1-3GalNAc.

β-elimination, were not inhibitory at 100 μM. Porcine stomach mucin in its native form was not inhibitory as opposed to its oligosaccharides obtained by β-elimination (Table 2). Oligosaccharides obtained by alkaline-borohydride degradation, from asialofetuin, IgA and porcine stomach mucin, and containing Gal(β1-3)-2-acetamido-2-deoxy-1,5-anhydrogalactitol were fourfold more powerful inhibitors than *N*-acetylgalactosamine; the disaccharide Gal(β1-3)GalNAc, obtained after endo-glycosidase digestion, was 62 times more powerful an inhibitor than the simple sugar. The aglycone portion derived from fetuin, was not inhibitory at 100 μM.

Amaranthus leucocarpus lectin reacted with porcine stomach mucin oligosaccharide I and II (Fig. 1), thus suggesting that the substitution of *N*-acetylgalactosamine by Gal(β1-4)GlcNAc(β1-6) of glycan II is well tolerated by the lectin. It is noteworthy that the lectin did not react with a more complex structure such as mucin oligosaccharide III which contains *N*-acetylgalactosamine in a terminal position (as suggested by 500 MHz ^{1}NMR spectroscopy).

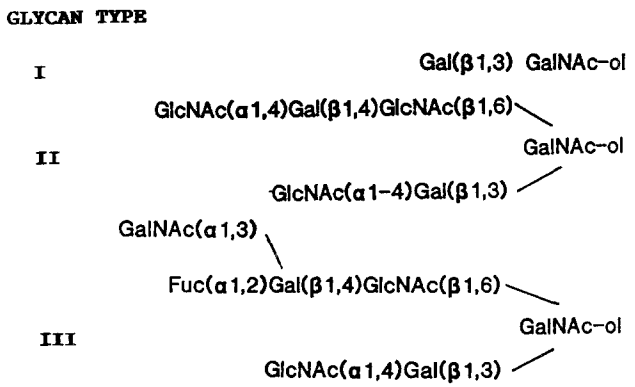

Figure 1. Structure of the oligosaccharide alditols from porcine stomach mucin used as inhibitors of the *Amaranthus leucocarpus* lectin.

Effect of pH and ionic strength

The lectin ability to agglutinate Pronase- and neuraminidase-treated human M and N erythrocytes was tested between pH 5 and pH 9. The optimal pH was found to be between pH 5.5 and pH 6.6; the hemagglutinating activity of M and N red cells diminished 99% and 75%, respectively, at pH 9. As far as the ionic strength is concerned, similar results were obtained. NaCl concentrations below 0.05 M abolished the agglutinating activity of the lectin (Fig. 2).

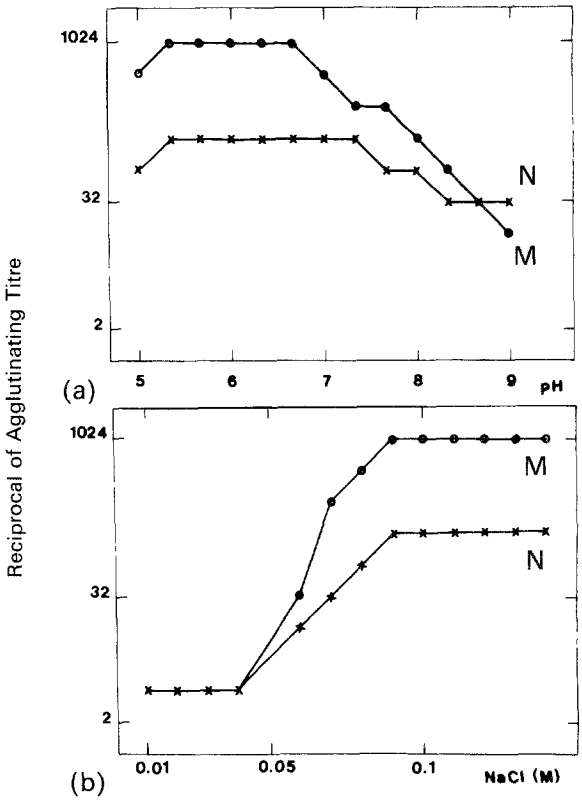

Figure 2. pH and salt dependence of the agglutinating activity of neuraminidase- and Pronase-treated N and M erythrocytes by *Amaranthus leucocarpus* lectin. The concentration of the lectin was 2.4 μg ml^{-1}. The agglutinating titre was assayed in the 'Universal' buffer at (a) different pH values, and (b) different NaCl concentrations.

Discussion

Our results show that *Amaranthus leucocarpus* lectin interacts specifically with the T-antigenic disaccharide Gal(β1-3)GalNAc and also recognizes *N*-acetylgalactosamine in the α anomeric configuration (T_n-antigen).

As for amaranthin, another lectin isolated from the seeds of *A. caudatus* [3], the most important loci for the *A. leucocarpus* lectin binding site are the hydroxyl group from the C-4 hydroxyl and the C-2 acetamido groups of the reduced *N*-acetylgalactosamine residue of the T-antigenic disaccharide. Similar to amaranthin [3] and jacalin, another anti-T lectin isolated from the seeds of the jackfruit *Artocarpus integrifolia* [22, 23], the combining site of the *Amaranthus leucocarpus* lectin seems to accommodate mainly the *N*-acetylgalactosamine residue found in the inner core of O-glycosidically linked glycans; for the three lectins, substitution(s) of the C-3 and/or the C-6 hydroxyl group(s) of the T_n-antigen with a galactose and/or an *N*-acetylneuraminic acid residue [16] did not decrease significantly the affinity of the lectins. Asialo-ovine submaxillary mucin was also a powerful inhibitor of the lectin agglutinating activity, due to its large number of accessible GalNAc α(1-3)Ser/Thr residues (T_n-antigen) [24].

The *Amaranthus leucocarpus* lectin combining site seems to be restricted to *N*-acetylgalactosamine residues situated in the inner core of the O-glycosidically linked glycan, since oligosaccharides obtained by alkaline-borohydride degradation from porcine stomach mucin or from fetuin and containing 2-acetamido-2-deoxy-1,5-anhydrogalactitol showed slightly less ability to inhibit the hemagglutinating activity of the lectin than the native glycoprotein or the O-glycosidically-linked glycans released by endo-*N*-acetylgalactosaminidase digestion. This was also observed for the *A. caudatus* lectin [3]. This suggests that the spatial feature provided by the O-glycosidic linkage plays an important role in the stabilization of the lectin interaction, as has been observed with *V. graminea* agglutinin [25].

We also found, as previous reports have indicated [6, 8], that *Amaranthus leucocarpus* lacks red blood cell group specificity, including the *N*-acetylgalactosamine-containing P or S phenotypes [26]. However, it is noteworthy that desialylated and mild Pronase-treated human type A, B and O erythrocytes with the M phenotype interact more strongly with the lectin than asialo-N red blood cells. The mild enzyme treatment avoided the loss of M or N antigen on the cell surface [27].

The interaction of *Amaranthus leucocarpus* with the erythrocyte receptors is ionic strength- and pH-dependent;

a decreased protonization of the glycopeptide receptors, induced by the pH increase, seems to reduce these interactions. Because the pH dependence was distinctively related to the type or phenotype of the erythrocytes, we consider that the decrease in binding activity is linked to the red blood cell receptors and not to the lectin. The pH dependence of the interaction between lectins and receptors has been studied for other lectins [25, 28–31]. So far the only clear dependence has been demonstrated with *V. graminea* agglutinin, suggesting the participation of the terminal leucine residue from the peptidic fragment of the N-antigen determinant (glycophorin-A^N [20]).

It has been established clearly that glycophorin possesses 13 structures, 12 are O-glycosidically linked and one is N-linked [32]. It is possible that the lectin receptors are glycophorin A^M molecules which possess three oligosaccharide chains attached to their terminal amino acid residues 2–4 [33]. Nevertheless, it is also possible that Pronase-treatment removed blood group M determinants [27] from the cell surface and the stronger agglutination of these cells may have depended on other undefined M-associated Pronase-resistant structures including glycolipids, or remaining O-glycosidically linked structures. But, similar to *Vicia villosa* lectin B_4, a T_n and CAD blood group specific lectin [34], the interaction is probably directed to glycoprotein rather than to glycolipid antigens.

To sum up, *Amaranthus leucocarpus* lectin possesses some structural similarities with other *Amaranthus* lectins [2–6]; these resemblances include the sugar specificity, but we have shown that there are subtle differences between the *A. leucocarpus* and *A. caudatus* lectins which could account for their different biological activities, such as lymphocyte mitogenic activity [2, 6, 8].

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