The Hydrophobic Character of Peanut (*Arachis hypogaea*) Isoagglutinins

Blanca Ortíz,[†] Macario Bacilio,[‡] Patricia Gorocica,[†] Luis Felipe Montaño,[§]
Yonathan Garfias,[†] and Edgar Zenteno*,[#]

Departamento de Bioquímica, Instituto Nacional de Enfermedades Respiratorias,
Tlalpan D.F., 14080 Mexico; Centro de Investigaciones Biologicas del Noroeste, La Paz,
Baja California Sur, 23000 Mexico; Departamento de Biologia Celular, Instituto Nacional de Cardiologia,
Tlalpan D.F., 14080 Mexico; and Laboratorio de Inmunologia, Departamento de Bioquimica,
Facultad de Medicina, UNAM, P.O. Box 70159, México 04510 D.F., Mexico

Peanut seed lectin (PNA) is widely used to identify tumor-specific antigens on the eukaryotic cell surface. In this work PNA was purified by affinity chromatography, using a column containing glutaraldehyde-treated human erythrocytes, whereas PNA isoforms were purified by hydrophobic interaction chromatography using Phenyl-Sepharose. The affinity-purified PNA and its isoforms consist of four equal subunits of 24.5 kDa each, all of which agglutinated human sialidase-treated erythrocytes equally well; however, differences in their relative thermostabilities and sugar specificities for lactose were observed. Fractions PNA-I and PNA-II possess higher affinity for lactose residues than the more hydrophobic isoforms III and IV. These findings suggest that the differences observed in PNA isoagglutinins are due to hydrophobic regions of the protein that influence the three-dimensional organization of the molecule as well as its thermal stability and sugar specificity.

INTRODUCTION

Peanut agglutinin (PNA) is becoming increasingly important due to its capacity to bind immature cortical thymocytes (Reisner et al., 1976; Sharon and Lis, 1990) and mature and activated CD8+ lymphocytes (Galvan et al., 1998) and to recognize specifically the T antigen (Galβ1,3GalNAcα1,0Ser/Thr) found in sialidase-treated human ABO erythrocytes (Lotan et al., 1975); it has been also useful in the identification of the modulator role of T antigen-containing receptors in macrophages (Maldonado et al., 1994). The agglutinin specificity of PNA for terminal β-D-galactosyl residues has enabled its purification by chromatography on Sepharose 6B, affinity chromatography on Sepharose-coupled ϵ-aminocaproyl-β-D-galactopyranosylamine, agar polyacrylamide beads, lactosaminyl AE-P-150, and lactosyl-Sepharose (Baues and Gray, 1977; Fish et al., 1978; Sutoh et al., 1977). Previous papers have suggested molecular heterogeneity within various purified PNA preparations (Dumont and Nardelli, 1979; Miller, 1983; Pueppke, 1981). Other studies have shown that the heterogeneity of the isolectin population in peanut varieties is basically due to the dissimilar composition of the isolectins' subunits, which show differences in physicochemical characteristics and in biological properties, that is, mitogenic acitvity (Miller, 1983; Pueppke,

1981). This paper reports on the isolation of the PNA isolectins by hydrophobic interaction chromatography and on the PNA hydrophobicity, determined by the lectin's tendency to adsorb to a hydrophobic adsorbent.

EXPERIMENTAL PROCEDURES

Lectin Purification. *Arachis hypogaea* (cultivar Georgia PV-1995) seeds were obtained in Tulyehualco (Mexico). The seeds were ground to a fine powder in a grinder, and the seed meal was skimmed with petroleum ether. The soluble proteins of 10 g of seed meal were extracted by agitation for 12 h with 10 volumes of 0.14 M NaCl at 4 °C. The pH of the extract was adjusted to 4 with 1 M acetic acid, and the extract was allowed to stand for 12 h at 4 °C, before the crude extract was obtained by centrifugation at 3000*g* for 10 min. The lectin was purified from the crude extract by affinity chromatography using a column containing glutaraldehyde-treated human O erythrocyte membranes (stroma) entrapped physically on Sephadex G-25 (Sigma Fine Chemicals, St. Louis, MO) prepared according to the method of Hernandez et al. (1993). Before being mixed with the Sephadex G-25, the glutaraldehyde-treated stroma was desialylated by incubation at 100 °C for 1 h in the presence of 0.02 N sulfuric acid, as described by Spiro and Bhoyroo (1974). The column was equilibrated with phosphate-buffered saline (PBS; 0.01 M sodium phosphate, 0.14 M sodium chloride, pH 7.2) before the crude extract (containing 160 mg of protein) was applied onto the stroma column (25 × 1.2 cm). Unretained material was eluted with PBS at a flow rate of 15 mL/h, until the A_{280} of the collected 1.5 mL fractions was below 0.01. Bound lectin (PNA) was eluted with 3% acetic acid, and the pH of each collected fraction was adjusted to 6 with 1 M NaOH; alternatively, the lectin was eluted with 200 mM lactose in PBS. Comparison of both methods showed the same protein yield. The hemagglutinating activity, in the presence of desiaylated human erythrocytes, was tested in each fraction.

Purification of PNA Isoforms. Hydrophobic chromatography was performed in a Phenyl-Sepharose CL-4B (Pharma-

* Address correspondence to this author at the Departamento de Bioquimica, Facultad de Medicina UNAM, P.O. Box 70159, 04510 México D.F., Mexico (fax 52.56.16.24.19; e-mail ezenteno@servidor.unam.mx).
† Instituto Nacional de Enfermedades Respiratorias.
‡ Centro de Investigaciones Biologicas del Noroeste.
§ Instituto Nacional de Cardiologia.
# UNAM.

6268   *J. Agric. Food Chem.,* Vol. 48, No. 12, 2000

Ortíz et al.

cia Biotechnology, Uppsala Sweden) column (13 × 1.6 cm) equilibrated with PBS/0.5 M ammonium sulfate at 22 ± 3 °C. Sixteen milligrams of purified PNA in 3 mL of equilibrium buffer was applied to the column at a 15 mL/h flow rate. Unretained material was extensively washed with the equilibrium buffer, and the lectin isoforms were eluted from the column into four fractions by sequential elution with PBS containing 0.25, 0.12, and 0.06 M or no ammonium sulfate (Zenteno et al., 1991). The A_{280} of the collected fractions and the hemagglutinating activity, in the presence of desialylated human erythrocytes type O, were determined in each 1.5 mL collected fraction. Each collected fraction was pooled and rechromatographied in the Phenyl-Sepharose column to obtain a better separation of each lectin isoform before its chemical characterization was performed. To confirm the hydrophobic interaction, we used Octyl-Sepharose CL-4B as hydrophobic matrix (Pharmacia) (13 × 1.6 cm) in similar conditions as Phenyl-Sepharose.

Analytical Methods. Protein concentration was determined according to the method of Bradford with Coomassie Blue R-250, using bovine serum albumin as standard (Bradford, 1976). Polyacrylamide gel electrophoresis (SDS−PAGE) was performed on a slab gel apparatus according to the method of Laemmli (1970). The amino acid analysis was performed on samples containing 100 μg of protein, hydrolyzed under vacuum with 2 mL of 6 M 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 the method of Bidlingmeyer et al. (1984), using norleucine as internal standard. There was no difference in amino acid composition among the three different hydrolysis incubation times.

Thermal Stability. The effect of temperature on the agglutinating activity of each purified fraction, previously dialyzed against PBS, pH 7.2, was tested by incubating the fractions at different time lengths (from 1 to 60 min) and temperatures (from 22 to 65 °C) in a water bath. Results are expressed as the percentage of hemagglutinating activity after incubation and compared to a lectin aliquot incubated at room temperature (22 ± 3 °C) (Hernandez et al., 1993). Experiments were performed in triplicate, and no differences were identified among the results obtained.

Sugar Specificity. The hemagglutinating activity of each fraction obtained through the PNA purification procedure was assayed in microtiter U plates (Nunc, Roskilde, Denmark) by using the 2-fold serial dilution procedure. The agglutinating activity was tested with a 2% (w/v) erythrocyte suspension in PBS. The erythrocytes were previously treated with 0.1 unit of *Clostridium perfringens* sialidase per 0.5 mL of packed erythrocytes at 37 °C for 30 min. The hemagglutinating titer is reported as the inverse of the last dilution with agglutinating activity. The sugar specificity of the PNA and each purified isoform was determined by comparing the effect of lactose, at different concentrations, on the hemagglutination induced by each lectin against sialidase-treated human O erythrocytes (Perez-Campos et al., 1997).

RESULTS

Purification of PNA and Isoforms. Purification of PNA was obtained by affinity chromatography on a column containing desialylated human O erythrocytes. The lectin eluted by the addition of either 3% acetic acid or 200 mM lactose to the chromatographic medium agglutinated sialidase-treated human erythrocytes, and its specific activity was 54 times higher than that of the crude extract. Affinity-purified PNA was applied to a Phenyl-Sepharose column and, by diminishing the salt concentration from the chromatographic medium, four fractions were eluted. Fractions I, II, and III were obtained with 0.25, 0.12, and 0.06 M ammonium sulfate concentrations, respectively; one more fraction (fraction IV) was eluted with distilled water (Figure 1). The purified isoforms correspond to 26.5, 25, 22, and 2%
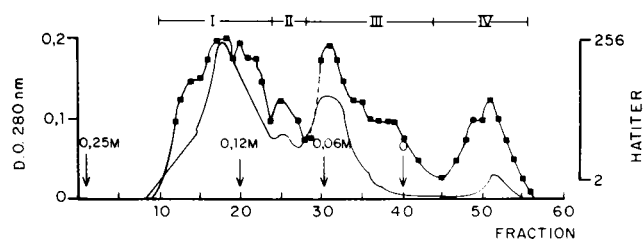

Figure 1. Purification of PNA isoforms by hydrophobic interaction chromatography. Crude extract (160 mg) was applied onto a column containing Phenyl-Sepharose 4B-CL, equilibrated with 0.5 M ammonium sulfate. Fractions I−IV were eluted by decreasing the ammonium sulfate concentration (dotted line). The OD at 280 nm (continuous line) and the hemagglutinating activity in the presence of sialidase-treated human erythrocytes type O (■) were tested.

Table 1. Purification Process of Peanut (*A. hypogaea*)[a] Agglutinin (PNA) and Isoforms

fraction	protein (mg)	HAU[b]	specific activity[c]
crude extract	972	44800	46
affinity chromatography			
unretained fraction	75.8	0	0
PNA	13.6	34072	2505
hydrophobic interaction chromatography			
PNA I	3.6	7871	2186
PNA II	3.4	6440	1894
PNA III	3.0	6371	2124
PNA IV	0.3	1056	3520

[a] From 10 g of seed meal. [b] Hemagglutinating units (HAU) with human erythrocytes type O treated with *C. perfringens* sialidase. [c] HAU/mg of protein.

(fractions I, II, III, and IV, respectively) of the total PNA protein purified by affinity chromatography (Table 1). All four fractions showed hemagglutinating activity toward desialylated human red blood cells. The yield of this activity corresponded to 64% of the total hemagglutinating activity initially added to the hydrophobic interaction chromatography (Table 1); fraction IV, which is the most hydrophobic, showed the highest specific activity in the presence of desialylated erythrocytes. None of the PNA isoforms showed hemagglutintaing activity toward native erythrocytes. Similarly to Phenyl-Sepharose, the Octyl-Sepharose column was also capable of retaining PNA; however, all of the lectin was recovered as a single fraction in the absence of ammonium sulfate, and no PNA isoforms were eluted by decreasing the salt concentration (data not shown).

Chemical Characterization. The PNA purified by affinity chromatography as well as the four purified isoforms proved to be homogeneous by SDS−PAGE; all fractions purified by hydrophobic interaction chromatography showed identical subunit molecular weights of 24.5 kDa (Figure 2). The amino acid compositions were very similar among the purified agglutinin and its isoforms. The main amino acids identified were Asp, Val, Ser, Gly, and, in minor concentrations, Met and His; Cys residues were not identified (Table 2). It was interesting to note that the amount of polar amino acids was slightly lower in the more hydrophobic isoforms; however, the amount of hydrophobic amino acids such as Phe, Leu, and Lys was increased in the most hydrophobic fractions. The data were similar in the three different hydrolysis incubation times in the amino acid determination.

Thermal Stability. The effect of temperature on the hemagglutinating activity of each fraction is sum-
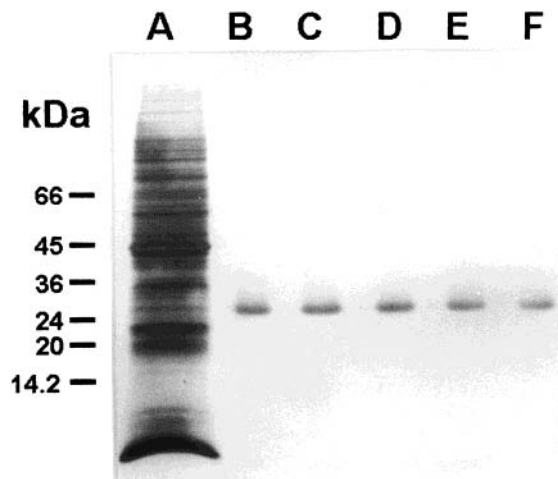
Peanut Isoagglutinins

J. Agric. Food Chem., Vol. 48, No. 12, 2000 **6269**

Figure 2. SDS–PAGE of the purified PNA isoforms: (A) crude extract (50 μg); (B) PNA purified by affinity chromatography on desialylated human stroma; (C–F) PNA isoforms (I–IV) purified by hydrophobic interaction chromatography (5 μg each). Molecular weight markers: bovine serum albumin (66 kDa); ovalbumin (45 kDa); glyceraldehyde-3-phosphate dehydrogenase (36 kDa), trypsinogen (24 kDa); trypsin inhibitor (20 kDa); α-lactalbumin (14.2 kDa).

Table 2. Amino Acid Composition (Residues per Mole) of the Purified PNA and Isolectins[a]

	lectin isoforms				
residue	PNA	FI	FII	FIII	FIV
Asp	28.6	28.8	26.6	25.9	25.6
Glu	15.8	14.6	15.6	16.3	16.4
Ser	19.9	19.7	19.9	19.5	18.4
Gly	19.8	19.9	19.5	19.9	18.5
His	1.2	1.4	1.2	1.2	1.1
Arg	7.8	7.0	7.0	7.3	6.5
Thr	13.1	13.9	13.9	12.7	12.4
Ala	13.1	13.4	14.4	15.2	15.9
Pro	14.1	15.9	13.5	12.2	13.2
Tyr	4.2	4.2	4.2	4.6	4.2
Val	25.5	25.0	25.3	25.5	25.9
Met	2.7	2.8	2.7	2.5	2.5
Cys	0	0	0	0	0
Ile	17.1	17.2	17.0	16.9	16.9
Leu	16.5	14.6	14.8	15.7	17.5
Phe	6.5	7.0	6.7	8.3	8.5
Lys	15.9	16.1	15.9	17.0	16.8

[a] Considering a molecular weight of 24.5 kDa.

Table 3. Effect of Temperature on Hemagglutinating[a] **Activity of PNA Isolectins**

temp (°C)	time (min)	FI	FII	FIII	FIV
30	30	100	100	100	100
45	30	75	75	50	50
55	15	25	25	12.5	0
55	30	6	0	0	0
65	1	75	75	50	50
65	10	25	12.5	0	0

[a] Reported values represent the percent of the hemagglutinating activity when compared with aliquots incubated at 22 ± 3 °C. Tests were performed with 35 μg/mL of PNA using sialidase treated human O erythrocytes.

marized in Table 3. Incubation of PNA or any of its purified isoforms at 65 °C for 15 min abolished completely the activity. Incubation of fractions I and II at 55 °C for 15 min diminished by 75% the initial activity; however, under the same conditions, the hemagglutinating activity of fractions III and IV was practically abolished.
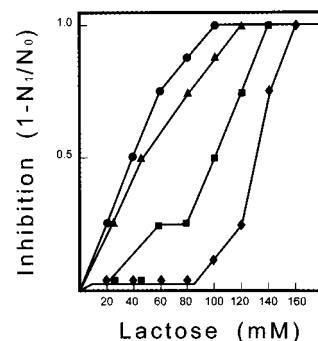

Figure 3. Specificity of purified PNA isolectins for lactose. PNA isoforms I (●), II (▲), III (■), and IV (◆), at 32 μg/mL concentration, were incubated for 30 min with different lactose concentrations before a suspension of 2% desialylated human O erythrocytes was added. Ni, hemagglutinating titer in the presence of lactose; No, hemagglutinating titer in the presence of PBS as control experiments.

Sugar Specificity. PNA and its purified isoforms agglutinated equally well desialylated human O erythrocytes. As indicated in Figure 3, the purified isoforms showed differences in their capacities to interact with their specific receptor on the erythrocyte membrane, although the four lectin isoforms were completely inhibited by 200 μM lactose. The lactose concentration required to inhibit 50% of the hemagglutinating activity of fractions I and II was 2-fold lower than the concentration necessary to inhibit fractions III and IV.

DISCUSSION

We purified the PNA by affinity chromatography using a column containing glutaraldehyde-treated human erythrocytes. The PNA isoforms were obtained by hydrophobic chromatography interaction, using a Phenyl-Sepharose column. Preliminary results had indicated that 100% of the hemagglutinating activity was adsorbed to the column only when the eluent contained at least 0.37 M ammonium sulfate, indicating that the adsorption induced by the presence of ammonium sulfate could be due to the fact that hydrophobic interactions are promoted by salts (Porath et al., 1973; Zenteno et al., 1991). The affinity-purified PNA and its isoforms indicated that they possess identical subunit molecular weights. Previous papers have suggested that molecular heterogeneity within various purified PNA preparations (Miller, 1983) is basically due to the dissimilar composition of the isolectins' subunits. Although each subunit has essentially the same characteristic tertiary fold that is found in other legume lectins, the structure of PNA exhibits an unusual quaternary arrangement of subunits (Banerjee et al., 1994, 1996). This structure also showed differences in subunit arrangement, which could be due to factors intrinsic to the protein molecule (Banerjee et al., 1994) and could explain in part not only the capacity to interact with a hydrophobic matrix but also the ability to accommodate to its specific ligand in complex structures among the cellular surface.

PNA not only recognizes the T-antigen (Galβ1,-3GalNAc) but also recognizes *N*-acetyllactosamine (Galβ1–4GlcNAc) and lactose(Galβ1–4Glc) (Lotan et al., 1975; Reisner et al., 1976), which are present at the termini of several cell-surface glycoconjugates (Varki, 1993). In this work we identified that the hemagglutinating activity of PNA and its isoforms interacts with

lactose; however, they showed slight differences in the interaction with this disaccharide. Fractions PNA-I and PNA-II possess higher affinity for lactose residues than the more hydrophobic isoforms PNA-III and PNA-IV, because the latter required 2-fold higher lactose concentrations to inhibit 50% of their hemagglutinating activity. These findings suggest that differences in hydrophobicity as well as in sugar specificity activity are due to the fact that isoforms could occur in a different three-dimensional organization (Banerjee et al., 1996).

The lectin isoforms showed differences in thermal stability. The temperature of maximal stability of the peanut agglutinin tetramer at pH 7.4 has been calculated to be ∼33 °C (Reddy et al., 1999); our results indicated that the isolectins with the lowest capacity to interact with a hydrophobic matrix are more stable at this temperature, confirming that the PNA isoagglutinins are proteins that show relevant differences in their three-dimensional organization. In summary, our findings strongly suggest that the heterogeneity of PNA should be represented by different rates of hydrophobic interaction between these biomolecules and hydrophobic ligands. Furthermore, we suggest that hydrophobic interaction chromatography could be a useful tool to identify molecular isoforms that might be expressed with a different structural organization.

LITERATURE CITED

Banerjee, R.; Mande, S. C.; Ganesh, V.; Das, K.; Dhanaraj, V.; Mahanta, S. K.; Suguna, K.; Surolia, A.; Vijayan, M. Crystal structure of peanut lectin, a protein with an unusual quaternary structure. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 227−231.

Banerjee, R.; Das, K.; Ravishankar, R.; Suguna, K.; Surolia, A.; Vijayan, M. Conformation, protein-carbohydrate interactions and a novel subunit association in the refined structure of peanut lectin-lactose complex. *J. Mol. Biol.* **1996**, *259*, 281−296.

Baues, R. J.; Gray, G. R. Lectin purification on affinity columns containing reductively aminated disaccharides. *J. Biol. Chem.* **1977**, *252*, 57−60.

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

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

Dumont, F.; Nardelli, J. Peanut agglutinin (PNA)-binding properties of murine thymocyte subpopulation. *J. Immunol.* **1979**, *37*, 217−224.

Fish, W. W.; Hamlin, L. M.; Miller, R. L. The macromolecular properties of peanut agglutinin. *Arch. Biochem. Biophys.* **1978**, *190*, 693−698.

Galvan, M.; Murali-Krishna, K.; Ming, L. L.; Baum, L.; Ahmed, R. Alterations in cell surface carbohydrates on T cells from virally infected mice can distinguish effector/memory CD8+ T cells from naive cells. *J. Immunol.* **1998**, *161*, 641−648.

Hernandez, E.; Ortiz, R.; Lopez, F.; Martinage, A.; Masso, F.; Montaño, L. F.; Zenteno, E. Purification and characterization of a galactose-specific lectin from *Psilocybe barrerae*. *Phytochemistry* **1993**, *32*, 1209−1211.

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

Lotan, R.; Skutelsky, E.; Danon, D.; Sharon, N. The purification, composition, and specificity of the anti-T lectin from peanut (*Arachis hypogaea*). *J. Biol. Chem.* **1975**, *250*, 8518−8523.

Maldonado, G.; Porras, F.; Fernandez, L.; Vazquez, L.; Zenteno, E. Effect of lectins on mouse peritoneal macrophage phagocytic activity. *Immunol. Invest.* **1994**, *23*, 429−436.

Miller, R. L. Purification of peanut (*Arachis hypogaea*) agglutinin isolectins by chromatofocusing. *Anal. Biochem.* **1983**, *131*, 438−446.

Perez-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.

Porath, J.; Sundberg, L.; Fornstedt, N.; Olson, I. Salting-out in amphiphilic gels as a new approach to hydrophobic adsorption. *Nature* **1973**, *245*, 465−466.

Pueppke, S. G. Multiple molecular forms of peanut lectin: classification of isolectins and isolectin distribution among genotypes of the genus *Arachis. Arch. Biochem. Biophys.* **1981**, *212*, 254−261.

Reddy, G. B.; Bharadwaj, S.; Surolia, A. Thermal stability and mode of oligomerization of the tetrameric peanut agglutinin: a differential scanning calorimetry study. *Biochemistry* **1999**, *38*, 4464−4470.

Reisner, Y.; Linker-Israeli, M.; Sharon, N. Separation of mouse thymocytes into two subpopulations by the use of peanut agglutinin. *Cell Immunol.* **1976**, *25*, 129−134.

Sharon, N.; Lis, H. Legume lectins—a large family of homologous proteins. *FASEB J.* **1990**, *4*, 3198−3208.

Spiro, R. G.; Bhoyroo, V. D. Structure of the O-glycosidically linked carbohydrate units of fetuin. *J. Biol. Chem.* **1974**, *249*, 5704−5717.

Sutoh, K.; Rosenfelf, L.; Lee, Y. C. Isolation of peanut lectin by affinity chromatography on polyacrylamide-entrapped guar beads and polyacrylamide (co-allyl α-D-galactopyranoside). *Anal. Biochem.* **1977**, *79*, 329−337.

Varki, A. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* **1993**, *3*, 97−130.

Zenteno, E.; Ochoa, J. L.; Montaño, L. F.; Debray, H.; Montreuil, J. *Machaerocereus eruca* cactus isolectins: Purification and characterization. *Plant Sci.* **1991**, *77*, 11−19.