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Machaerocereus eruca cactus isolectins. Purification and characterization

Edgar Zenteno[a], Jose Luis Ochoa[b], Luis Felipe Montaño[c], Henry Debray[d] and Jean Montreuil[d]

[a]*Depto. Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, Ap. Postal 70159 and CIB, Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos,* [b]*Centro de Investigaciones Biológicas de la Paz, B.C.S.,* [c]*Lab. Immunobiología, Depto. Bioquímica, Instituto Nacional de Enfermedades Respiratorias, 14080 (México) and* [d]*Laboratoire de Chimie Biologique, Université des Sciences et Techniques de Lille Flandres Artois, Villeneuve d'Ascq 59655 (France)*

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Four isolectins from the cactus *Machaerocereus eruca* (ME-C2, D2, E2, F2) have been isolated and purified by hydrophobic and affinity chromatography on a red blood cell stroma column; in their native form there were differences in the molecular weights, whereas in their monomeric form they all showed a repetitive subunit of 35 kDa. The glycan portion of the isolectin which is composed of glucose, galactose, rhamnose, xylose and mannose, represented 20, 24, 26 and 30% of their weight, for ME-C2, D2, E2 and F2, respectively. All four isolectins were closely related in amino acid composition, although the less hydrophobic isolectin contained more glycine and threonine than the more hydrophobic fraction. Hapten inhibition assays indicated that the isolectins possess specificity for D-galactose, *N*-acetyl-D-galactosamine and galactose-containing glycoproteins and glycopeptides. All the isolectins were capable of suppressing the murine humoral immune response to particulated antigens, although no mitogenic activity in murine lymphocytes was found. The differences found in their physicochemical properties, glycan portion, ability to bind to a hydrophobic gel and amino acid composition suggest that either they are the products of related but distinct genes or the result of different post translational protein processing.

Key words: plant lectins; cactus lectins; *Machaerocereus eruca*; hydrophobic chromatography

Introduction

Lectins are proteins or glycoproteins that selectively recognize and bind carbohydrate residues [1]. This property makes them useful in blood group typing [2], carbohydrate structural analysis [3,4] and glycoconjugate isolation and characterization [5,6]. We have recently purified by affinity chromatography on porcine stomach mucin two lectins from the cactus *Machaerocereus eruca* [7] with similar chemical composition and ability to recognize human erythrocytes. One of these lectins was eluted with water from the affinity matrix [7] suggesting the participation of hydrophobic interactions.

Bearing the latter in mind, we report a new method for the isolation of *M. eruca* isolectins that combines hydrophobic interaction chromatography and affinity chromatography on an immobilized human red blood cell stroma column. In order to identify the nature of the interaction mechanisms of the isolectins with sugars, hapten inhibition assays with isolated *O*-glycosidically linked glycans and *N*-glycosylpeptides were carried out. We also tested the ability of the isolectins to influence the immune response and in order to identify differences in their physicochemical properties, other than their capacity to interact with a hydrophobic matrix, we compared the effect of divalent cations and temperature on their hemagglutinating activity.

Correspondence to: Edgar Zenteno, Depto. de Bioquímica, Facultad de Medicina, U.N.A.M., Apdo. Postal 70-159, México 04510.

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Material and Methods

Material

The plant cactus *M. eruca* was collected in Puerto San Carlos, Baja California Sur, Mexico and identified at the Centro de Investigaciones Biologicas de Baja California Sur, Mexico [8]. Sephadex G-25, G-200, Phenyl-Sepharose CL-4B, agarose and molecular weight markers were obtained from Pharmacia Fine Chem. (Uppsala, Sweden); Ultrogel ACA-202 and Biogel P-2 were from IBF-Biotechnics (Clichy, France). Jack bean meal β-galactosidase from *Canavalia ensiformis* was a gift from Dr. S. Bouquelet. Human sero and lactotransferrin, and human IgA and IgG were a kind gift from Dr. G. Spik. Hen ovomucoid was donated by Dr. B. Fournet, all at the Universite des Sciences et Techniques de Lille Flandres Artois, Villeneuve d'Ascq, France. All the remaining chemicals, sugars and proteins were obtained from Sigma Chemical Co. (St. Louis, MO).

Extraction procedure

The juice of 1 kg of cactus stem was obtained with a food processor and was centrifuged at 3000 × *g* for 15 min at 4°C. Proteins in the supernatant fluid were precipitated by addition of solid ammonium sulfate to a final concentration of 66% (w/v). The precipitate was collected by centrifugation, dialyzed against 5 mM citrate buffer (pH 5.5) and solid ammonium sulfate was added to a final concentration of 0.5 M before storing. Aliquots were kept at 4°C until used.

Chromatographic procedures

Hydrophobic interaction chromatography was carried out on a Phenyl-Sepharose CL-4B column (12 × 2.8 cm) at room temperature. The column was equilibrated with 5 mM citrate buffer (pH 5.5) containing 0.5 M ammonium sulfate. A suspension of 160 mg of the precipitated fraction in 0.5 M ammonium sulfate was poured onto the column which was thoroughly washed with the equilibration buffer to obtain unadsorbed *M. eruca* fractions A and B, both of which lacked hemagglutinating activity. In order to elute the adsorbed agglutinating activity from the column, sequential elutions with citrate buffer containing 0.25 M, 0.12 M, 0.06 M and no ammonium sulfate were performed. The protein content of the fractions thus obtained was monitored at 280 nm and their hemagglutinating activity tested. Those fractions with hemagglutinating activity were dialyzed against phosphate buffered saline (0.02 M sodium phosphate and 0.15 M sodium chloride, pH 7.4) (PBS) and poured onto a column (2.8 × 12 cm) containing human type O red blood cell stroma physically immobilized on Sephadex G-25, previously equilibrated with PBS at room temperature [9]. The elution of the active material was performed with distilled water at room temperature at a 14-ml/h flow rate.

Analytical methods

Protein concentration was determined by the method of Lowry [10] with bovine serum albumin as standard. Carbohydrate content was determined by the phenol-sulphuric method [11] with lactose as standard. One ml of purified isolectins (125 μg/ml) were treated with 30 μl of 0.3% (w/v) phenol to prevent degradation of tyrosine residues before being subjected to hydrolysis for 72 h under vacuum with 2 ml of 6 N HCl at 110°C in sealed containers and its amino acid composition determined in a Beckman 119 CL amino acid analyzer. The quantitative composition of sugars was determined by gas chromatography as trifluoracetyl alditol derivatives after methanolysis, using D-mannitol as an internal standard [12]; experiments were performed on a 5% silicone OV-210 column (Applied Science Lab., U.S.A.) in the 100—240°C temperature range at 1°C/min on a Varian 2100 gas chromatograph (Orsay, France).

The isolectin molecular weight was determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 5—25% gradient gels according to Laemmli [13]; the gel was stained according to Oakley et al. [14] with the Bio-Rad silver stain kit (Bio-Rad, CA). To determine the isolectin molecular weight we used Bio-Rad SDS-PAGE low molecular weight standards (Bio-Rad, CA). The native lectin molecular weight was determined by ultracentrifugation experiments in linear 5—30% sucrose gradients using a Beckman LS-65 ultracentrifuge (Beckman Instruments, U.S.A.); molecular weight markers included myoglobin (17

200), bovine serum albumin (68 000), human immunoglobulin G (150 000) and catalase (240 000). The sedimentation coefficient was determined according to Martin and Ames [15] and the molecular weight according to Rendon and Calcagno [16].

Hemagglutination assays

Hemagglutinating activity was assayed in microtiter U plates (NUNC, Denmark) using 25 μl of 2% (w/v) glutaraldehyde-fixed human ABO erythrocytes in PBS according to the 2-fold serial dilution procedure [17]. The sugar specificity of the lectin was tested by the inhibition of the hemagglutinating activity [17]; results are expressed as the minimal concentration of sugars, glycoproteins, *O*-glycosidically-linked glycans and *N*-glycosylpeptides that effectively inhibited 4 hemagglutinating dose units of the lectin.

Preparation of glycans and glycopeptides

Stroma from human group O erythrocytes was obtained by the method described by Dodge et al. [18]; its lipid content was eliminated by sequential extraction using chloroform/methanol (2:1, v/v) three times followed by chloroform/methanol (1:2, v/v) three times. The glycoproteins thus obtained were dried with nitrogen. *O*-Glycosidically-linked glycans and *N*-glycosylpeptides from fetuin and human stroma were liberated by alkaline reductive treatment [19] and further purified by gel filtration chromatography on Ultrogel ACA-202 and Biogel P-2. Released *N*-glycosylpeptides from fetuin were desialylated by incubation at 100°C for 1 h in the presence of 0.1 M trifluoracetic acid; these asialo-*N*-glycosylpeptides were further chromatographed on Biogel P-2 and degalactosylated with Jack bean meal β-galactosidase [20].

Physicochemical properties

M. eruca purified isolectins (125 μg/ml in PBS) were exposed to different salt (NaCl) concentrations and, to a wide range of temperatures for various periods of time, before assessing their hemagglutinating activity. In order to determine the effect of cations on the isolectin's agglutinating activity we first incubated the isolectin with 1 M (v/v) acetic acid followed by treatment with 0.1 M (w/v) ethylenediamine tetraacetic acid (EDTA); after extensive dialysis against deionized water the isolectin activity was tested in the presence or absence of 0.05 M $MnCl_2$, $MgCl_2$ and $CaCl_2$.

Immunological assays

Six-week-old female CD-1 mice were injected intraperitoneally with 1—300 μg of each isolectin in 100 μl of 0.15 M NaCl two days later the animals were immunized with 0.5% sheep red blood cells (SRBC). On the fifth day the animals were bled and their heat inactivated serum tested for SRBC antibodies by a direct hemagglutination assay [21,22].

CD-1 mice spleen cells were obtained through standard procedures and resuspended to 4×10^6 cells/ml in RPMI-1640 suplemented with 10% fetal bovine serum. A quantity (0.1 ml) of each cell suspension was dispensed into multiple tissue culture plate wells (NUNC, Denmark) together with 0.1 ml of culture medium containing different concentrations of the lectin crude extract and the isolectins. Cultures were maintained for 72 h at 37°C in a humidified chamber with a 7% (v/v) CO_2 atmosphere and were pulsed with 1 μCi of [*methyl*-^{3}H]thymidine, (spec. act., 6.7 Ci/mmol, New England Nuclear, Boston, MA) for 18 h before harvest. Incorporated radioactivity was detected with a Beckman LS-6000 SE scintillation counter.

Ouchterlony's double immunodiffusion tests were performed in 1.5% agarose plates. Purified isolectins (10 μg) and 30 μg of crude extract were made to react with *M. eruca* immune rabbit sera, obtained by immunizing intraperitoneally 2-month-old New Zealand female rabbits with 0.4 mg of *M. eruca* isolectin C2 in 0.5 ml of Freund's complete adjuvant, weekly for 4 weeks. Immunodiffusion plates were stained with 0.1% (w/v) amido black in 3% (v/v) acetic acid in distilled water and extensively washed with 3% (v/v) acetic acid in PBS.

Results

Hydrophobic and affinity chromatography have been used to isolate isolectins of *M. eruca* (ME) from a fraction insoluble in 66% ammonium

sulfate (Table I). Four peaks which contained 80% of the hemagglutinating activity were eluted from the Phenyl-Sepharose column by step wise decrease in the ammonium sulfate concentration (Table I and Fig. 1). All four fractions were further purified by affinity chromatography on a red blood cell stroma column; the hemagglutinating activity was recovered from the column with distilled water (fractions numbered 2), protein eluted with PBS (fractions numbered 1) did not posses agglutinating activity (Table I). The specific activity of the isolectins thus obtained, as compared to the crude extract, was 59, 31, 21 and 23-fold higher for ME-C2, ME-D2, ME-E2 and ME-F2, respectively.

SDS-PAGE revealed that the four purified isolectins posses a similar subunit of 35 kDa (Fig. 2). Ultracentrifugation on sucrose gradients revealed that the molecular weight of the four isolectins was 163 000 (ME-C2), 165 000 (ME-D2), 170 000 (ME-E2) and 177 000 (ME-F2) with S_{w20} values of 7.2, 7.4, 7.5 and 7.7, respectively.

The amino acid composition of *M. eruca* isolectins is listed in Table II. As with many plant lectins, they lack cysteine and methionine residues, are rich in glycine and alanine residues, and are poor in arginine, tyrosine and histidine residues. The four isolectins showed some differences in carbohydrate composition (Table III) and the sugar content corresponded to 20% (ME-C2), 24% (ME-D2), 26% (ME-E2) and 30% (ME-F2) by weight.

Table I. Purification of *M. eruca* isolectins. Data based on 1 kg of *M. eruca* cactus stem.

Fraction	Total protein (mg)	Total HAU[a]	Spec. act.[b]
Crude extract	1680	415 000	0.2
66%$(NH_4)_2SO_4$ precipitate	1008	415 000	0.4
Hydrophobic chromatography			
A	273	0	0
B	111	0	0
C	193	126 681	0.6
D	126	78 850	0.6
E	82	62 250	0.7
F	163	66 400	0.4
Affinity chromatography			
C1	111	0	0
C2	9	106 240	11.8
D1	69	0	0
D2	11	67 811	6.1
E1	45	0	0
E2	13	54 116	4.1
F1	99	0	0
F2	14	63 910	4.5

[a]HAU, hemagglutinating units. Experiments were performed with human type O erythrocytes.
[b]Specific activity is reported as HAU per μg of protein.

All four isolectins reacted against a rabbit αME-C2 immune serum by immunodiffusion; the precipitation band was common to all the isolectins.

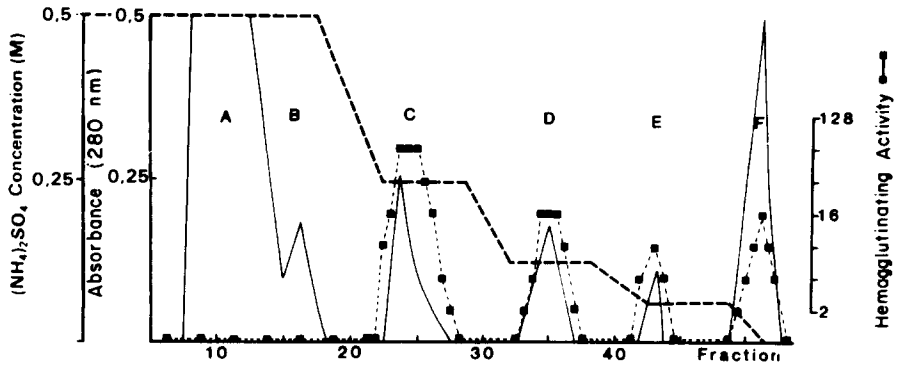

Fig. 1. Hydrophobic chromatography on a 12 × 2.8 cm Phenyl-Sepharose column of *M. eruca* 66% ammonium sulfate insoluble fraction. The continous line (—) represents the amount of protein detected by spectrophotometry at 280 nm, the dashed line (- - -) represents the ammonium sulfate step wise elution pattern and finally, the cactus' hemagglutinating activity against human type O erythrocytes, which was detected in peaks C, D, E and F, is represented by (■- -■).

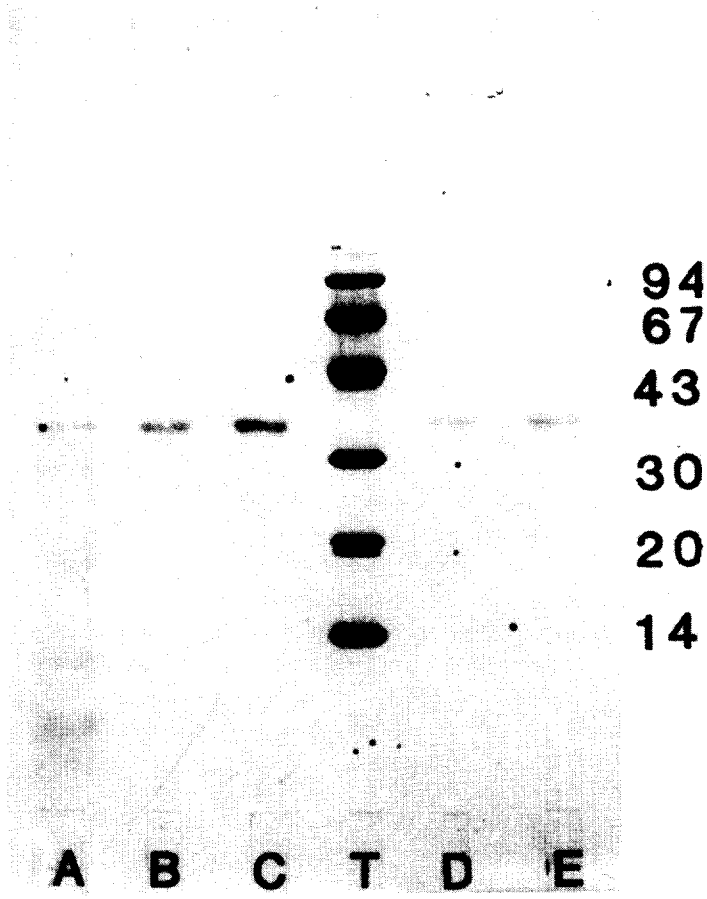

Fig. 2. SDS-PAGE of 5 μg of *M. eruca* isolectins. Lane A corresponds to the cactus crude extract, lanes B, C, D and E correspond to isolectins ME-C2, ME-D2, ME-E2 and ME-F2, respectively. Lane T corresponds to the molecular weight standards phosporylase B (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 000) and lysozyme (14 000).

ME-C2, ME-D2 and ME-E2 isolectins at concentrations of 1 μg/ml were capable of agglutinating human A, B and O erythrocytes, 16 μg/ml was required for ME-F2. The concentration of isolectin needed to agglutinate donkey erythrocytes was significantly lower, 0.01 μg/ml for ME-C2 and ME-D2, and 0.04 μg/ml for ME-E2 and ME-F2. Variations in pH, treatment with 1 M acetic acid and 0.1 M ethylenediamine tetraacetic acid (EDTA) or addition of $CaCl_2$, $MnCl_2$ or $MgCl_2$ did not affect the isolectin agglutinating activity whereas changes in ionic strength, represented by NaCl concentrations of 0.01 M for ME-C2 and ME-D2 and 0.001 M for ME-E2 and ME-F2, decreased this activity. It was interesting to observe that ME-E2 and ME-F2 lost their agglutinating activity when incubated at 80°C for 5 min; ME-C2 and ME-D2 showed a 25 and 12% decrease, respectively.

The isolectin sugar specificity was determined by hemagglutination inhibition assays using simple sugars, glycoproteins or their released *O*-glycosidically-linked glycans and *N*-glycosylpeptides. D-Glucose, D-mannose, D-ribose, D-fucose, L-rhamnose, maltose, *N*-acetyl-D-glucosamine and α-methyl-D-mannoside had no

Table II. Amino acid composition of *M. eruca* isolectins. The amino acid composition is expressed as the nearest integer of the number of residues per molecule, assuming a molecular weight of 35 000 for the isolectins.

Amino acid	Isolectin			
	ME-C2	ME-D2	ME-E2	ME-F2
Asx	29	26	28	30
Thr	28	26	18	18
Ser	33	33	31	28
Glx	24	27	27	28
Pro	14	14	13	15
Gly	61	60	55	51
Ala	40	38	40	40
1/2 Cys	0	0	0	0
Val	25	25	23	23
Met	0	0	0	0
Ile	9	9	10	12
Leu	20	21	23	25
Tyr	8	8	8	8
Phe	10	10	11	12
His	3	3	3	3
Lys	12	11	11	12
Arg	6	7	9	9

Table III. Carbohydrate composition of *M. eruca* isolectins. Results are based on 35 000 as the isolectin molecular weight. Arabinose, GalNAc, GlcNAc, sialic and glucuronic acid were not found.

Sugar	Composition (mol/mol)			
	ME-C2	ME-D2	ME-E2	ME-F2
Rhamnose	6	11	10	10
Xylose	11	14	8	8
Fucose	0	0	0	0.2
Galactose	2	6	6	8
Mannose	0.2	2	2	2
Glucose	20	37	30	46

inhibitory effect at concentrations as high as 250 mM, as opposed to D-galactose, lactose, *N*-acetyl-D-galactosamine, methyl α- or β-galactosides and *p*-nitrophenyl α- or β-galactosides which, at concentrations of 50 mM or less, inhibited 4 hemagglutinating units of each of the *M. eruca* isolectins.

Interestingly enough, ME-F2 was also inhibited by 100 mM L-fucose. A powerful inhibition of the hemagglutinating activity of the isolectins by more complex structures such as pig stomach mucin, bovine submaxilar mucin and fetal calf fetuin, was observed. Human serum IgG, human serotransferrin and other glycoproteins were also inhibitory (Table IV). *O*-Glycosidically-linked glycans from human erythrocytes stroma, fetuin and human IgA were also inhibitors, similar to released *N*-glycosylpeptides from human erythrocytes stroma and fetuin, but interestingly enough, fetuin's asialo *N*-glycosylpeptides were much more powerful inhibitors than its *N*-glycosylpeptide as opposed to fetuin's asialo-agalacto-*N*-glycosylpeptides which

Table IV. Minimal concentration (μM) of glycans, glycopeptides and glycoproteins capable of inhibiting 4 hemagglutinating units of *M. eruca* isolectins. The hemagglutinating activity was tested with normal type O human erythrocytes. N.I., not inhibitory at 100 mM. All isolectins were adjusted to 4 hemagglutinating units against human type O erythrocytes for these experiments.

Substrate	Isolectin			
	ME-C2	ME-D2	ME-E2	ME-F2
Hen ovomucoid	NI	NI	NI	NI
Pig stomach mucin	0.04	0.04	0.03	0.01
Bovine submaxilary mucin	0.08	0.04	0.02	0.02
Human orosomucoid	110	65	65	40
Calf fetuin	0.04	0.02	0.02	0.005
Fetuin-*N*-glycosylpeptides	58	58	27	27
Fetuin-asialo-*N*-glycosylpeptides	12	6	3	1.5
Fetuin-asialo-agalacto-*N*-glycosylpeptides	N.I.	N.I.	N.I.	N.I.
Fetuin *O*-glycan	10	5	5	1.5
Human stroma *N*-glycosyl-peptides	56	56	28	28
Human stroma *O*-glycan	18	9	9	4.5

were not inhibitors at concentrations as high as 100 μM.

M. eruca isolectins had no mitogenic effect on mouse spleen cells or human peripheral blood mononuclear cells compared with concanavalin A which gave a stimulation index of 26 and 31, respectively. All four isolectins at a concentration of 30 μg/0.1 ml PBS suppressed the mouse antibody response to sheep red blood cells provided that the isolectins were injected intraperitoneally two days before the particulate antigen.

Discussion

The purification of four isolectins from the plant cactus *M. eruca* has been obtained by the combination of hydrophobic interaction and affinity chromatography on a human erythrocyte stroma column. Previous reports indicated that *M. eruca* contained more than one lectin whose activity probably depended on ionic forces [7]. In this paper we confirm these previous observations. We were able to isolate four *M. eruca* isolectins by interaction with a hydrophobic matrix, and as the stroma column proved, by diminishing the ionic forces of the chromatographic media. This behavior has also been reported for *Eranthis hyemalis* root tuber [23], *Amaranthus leucocarpus* [24] and the *Phaseolus* spp. [9] lectins, pointing to the possibility that elution of the isolectins was possible because a hydrophobic portion of the molecule is located adjacent to the lectin active site, or as has been shown for Con-A [25], lima bean [26] and elderberry bark [27] lectins, hydrophobic forces participate in the lectin's recognition mechanisms.

The electrophoretic pattern of all four isolectins demonstrated a single band on SDS-PAGE of 35 kDa; ultracentrifugation experiments showed that the isolectin molecular weights varied from 163 to 177 kDa which indicates that the isolectins exist as tetramer molecules. The differences in the isolectin molecular weights seem to correspond to their rate of glycosylation since their amount of neutral sugars by weight varies from one to another (20% for ME-C2, 24% for ME-D2, 26% for ME-E2 and 30% for ME-F2). The amino acid composition of the isolectins is similar, the difference being in the relative concentration of some residues such as glycine, threonine, isoleucine, leucine, arginine and serine; similarly, the carbohydrate composition showed only quantitative differences. The similarity of all four isolectins is consistent with the observation that they all reacted to give a single confluent band when tested against ME-C2 immune serum (data not shown), a result which indicates the presence of similar epitopes in all four *M. eruca* isolectins.

The relative differences in the extent of isolectin glycosylation apparently confers different physicochemical properties. The most highly glycosylated isolectin, ME-F2, interacted more strongly with the hydrophobic matrix, was more heat-labile and was less dependent on the ionic strength than isolectin ME-C2 which is glycosylated to a lesser degree. The relationship between high glycosylation and capacity to be adsorbed to a hydrophobic matrix has already been reported with plant structural glycoproteins [28], with complex oligosaccharide chains [29] and with human immune interferon [30]. In this regard it was interesting to observe that the sugar composition of *M. eruca* isolectins seems to correspond to the xyloglucan and rhamnogalacturonan structural conforming polysaccharides of dicotyledonous plant primary cell walls [31,32] and not to the usual *N*-glycosidically-linked oligosaccharides of the complex or high mannose type reported in the majority of plant lectins. Nevertheless the participation of lectin binders [33] as associated ligands in the isolectin preparation, and their influence in the interaction with the hydrophobic matrix must be considered.

The hemagglutinating activity of the isolectins was not species-specific for any of the human blood groups tested in the ABO system. Hapten inhibition assays indicated that the four isolectins are specific for D-galactose, their derived glycosides and *N*-acetyl-D-galactosamine. The isolectins were also inhibited by *O*-glycosidically-linked glycans containing sialic acid, galactose and *N*-acetyl-D-galactosamine such as those isolated from fetuin and human stroma. However, human orosomucoid and both fetuin *N*-glycosylpeptide and human stroma also showed affinity for the lectin probably as a result of the interaction between

the former's structures and galactose as shown by the observation that glycoproteins devoid of this sugar, such as ovomucoid or fetuin asialo agalacto-*N*-glycosylpeptides did not inhibit the isolectin's hemagglutinating activity. Despite this it was interesting to observe that native glycoproteins were much better inhibitors than their released oligosaccharides, an observation already made with other lectins such as *Lens culinaris* agglutinin and *Griffonia simplicifolia* agglutinin II [34]. A possible explanation for this phenomenon could be that the linkage of amino acids to the glycan leads to a more rigid structure than that of the oligosaccharides [35].

The four *M. eruca* isolectins were not mitogenic to murine or human mononuclear cells but managed to suppress the mouse humoral immune response to particulate antigens. We are currently trying to determine the possible mechanism of action. In summary our results showed that *M. eruca* isolectins have closely related sugar specificity and similar biological activity, moreover, the differences found in their physicochemical properties and in their amino acid composition suggests that either they are the products of related but distinct genes or the result of different post translational protein processing.

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