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ISOLATION AND PARTIAL CHARACTERIZATION OF A LECTIN FROM *ARTOCARPUS INCISA* L. SEEDS

RENATO A. MOREIRA*, CARLOS C. CASTELO-BRANCO, ANA C. O. MONTEIRO, RICARDO O. TAVARES and LEILA M. BELTRAMINI

Laboratório de Lectinas, Departamento de Bioquímica e Biologia Molecular, Centro de Ciências, Universidade Federal do Ceará, Caixa Postal 6020, CEP 60451-970, Fortaleza (CE), Brazil; Departamento de Física e Informática, Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, (SP), Brazil

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Key Word Index—*Artocarpus incisa*; *Moraceae*; frutalin; isolation; lectin; D-Galactose-binding.

Abstract—A lectin was isolated from the saline extract of *Artocarpus incisa* seed by affinity chromatography on cross-linked *Adenanthera pavonina* galactomannan in 0.15 M NaCl. The lectin was also retained in a D-gal-agarose resin and had no requirements for divalent metal cations (Ca^{2+} and Mn^{2+}) for activity. The lectin contains 2.1% of carbohydrate and is characterized by high contents of acidic and hydroxylated amino acids. The lectin presented two protein bands in SDS-PAGE, with M_r 15.5 and 12 kDa, respectively, and contains no α-helix, 64% antiparallel β-sheet and 21% parallel β-sheet/β-turn. When submitted to gel filtration in Superose 12 R (FPLC) and Superdex 75 HR 5/5 (HPLC) columns, the lectin showed an M_r of 48–49 kDa, suggesting a tetrameric structure. © 1998 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

Lectins, carbohydrate-binding protein, are widely distributed in the Plant Kingdom, and leguminous seeds are a particularly rich source of them. Only a few lectins have been isolated from other families. Among these, in the *Moraceae* well characterized lectins have already been isolated from *Artocarpus integrifolia* [1–4] and *Maclura pomifera* [5]. A previous study has also reported the isolation and partial characterization of a lectin from *Artocarpus incisa* seeds, through affinity chromatography on AB(+) human stroma column [6]. The main characteristics of this class of protein are based on their ability to interact with carbohydrates and thus combine with glycocomponents of cell surface, leading to their biological properties. Although the most rational classification of the lectins is based on their respective specificity for monosaccharides [7, 8], the individual members of the groups may show appreciable differences in oligosaccharide specificity [9, 10]. Thus, even lectins belonging to the same sugar-binding specificity show differences in their interactions with cell membranes and glycoconjugates [11–15], although these differences are less pronounced when the lectin is within the same genus. These differences include mitogenic stimulation and γ-interferon production on human lymphocytes [16], macrophage stimulation and leukocyte accumulation [17], edematogenic effects [18] and histamine release [19]. The isolation and characterization of non-leguminous lectins is of importance in order to study why minor differences in the structure can lead to dramatic differences in biological properties, and even to investigate the ubiquitous nature of the lectins.

* Author to whom correspondence should be addressed.

RESULTS AND DISCUSSION

The plant

Artocarpus incisa L. is a wide spread plant, common in pan-tropical regions. In Brazil are found two types of *A. incisa*, the 'fruta pão de massa', an *apyrena* (a non-seminifera, no seeds) type, and the 'fruta pão de caroço', a *seminifera* type, containing up to 80 seeds in the fruit (with *ca* 2 kg). Both types are consumed, cooked, by the local population. The seed of *Artocarpus incisa* type *seminifera* shows a high content of water (up to 60%), a moderate content of protein (12.25% in dry basis), and a low content of lipid (Table 1).

Isolation of the lectin

The purification of *A. incisa* lectin was obtained by a simple methodology and is summarized in Table 2. The affinity chromatography step, on a column of

Table 1. Percent composition of the seed of *Artocarpus incisa*, calculated on a dry matter basis

Fraction	Content %
Crude protein*	12.3
Lipids	4.9
Fibres	2.5
Ash	3.6
Carbohydrates	76.8

* $N_2 \times 6.25$.

Adenanthera pavonina cross-linked galactomannan, is depicted in Fig. 1. The peak eluted with 0.2 M D-galactose, contained all the hemagglutinating activity and was named frutalin. Further rechromatography of the lectin on a column of D-galactose-agarose revealed one single, sharp and symmetrical retained peak, eluted with D-galactose 0.2 M.

Properties of frutalin

The lectin frutalin agglutinates human erythrocytes of the ABO system at a concentration of 0.2 μg per ml (Table 2). With rabbit erythrocytes, the lectin showed the same activity, but with other animals (cow, goat, pig and sheep) the activity was lower (data not shown). Although the lectin could be isolated by affinity chromatography on a galactomannan-containing matrix and eluted with D-galactose, its agglutinating activity with red blood cells was not inhibited by this sugar. This activity was inhibited only by asialofetuin (0.1 mg of asialo fetuin reduced the activity of 1 mg of frutalin to 4%). These results suggest a more complex structure for the carbohydrate specificity of the lectin, although involving D-galactose. Thus the lectin can be classified in the general group of D-galactose-binding lectins [8], is a hololectin and can be named, following Peumans proposal [20], as LC.P.Art.inc.1.31.1. Frutalin is not a metallo-protein, as the haemagglutinating activity was not affected by prolonged dialysis against 0.2 M EDTA, followed by dialysis against 0.15 M NaCl.

Structural aspects

The SDS/PAGE of frutalin, both in the absence and presence of 2-mercaptoethanol, showed two bands with M_r of 15.5 and 12 kDa (Fig. 2), respectively. The lectin, when dissolved in 0.15 M NaCl, had an absorption value at 280 nm and 1 cm cell ($A_{1cm,1\%}$) of 10.73. The carbohydrate content, determined by the method of Dubois [21], was 2.1%. The amino acid composition (Table 3) was characterized by a very low content of sulphur containing amino acids and a high content of acidic and hydroxylated amino acids. When compared by cluster analysis with those of the lectins from *Artocarpus integrifolia* and *Maclura pomifera*, these results show 88% similarity.

The M_r of frutalin, estimated by gel filtration on Superose 12R column, at pH 8.3, in the presence of D-galactose and Superdex 75 HR 5/5 column, with PBS, pH 7.4 in the presence of D-mannose was of 48 and 49 kDa, respectively, suggesting a tetrameric structure. Similar results were obtained by Young *et al.* [5] for the lectins of *A. integrifolia* (46 kDa) and *M. pomifera* (44 kDa).

By isoelectric focusing, frutalin showed a broad band with pI between 8.8 and 9.0, suggesting the existence of isoforms. When this value was compared with those obtained for the other lectins from the *Moraceae* [5], it was found to be slightly higher than that of jacalin (7.4–8.3) but much more basic than the lectin from *M. pomifera* (4.6–4.8).

The CD (circular dichroism) spectrum of frutalin (Fig. 3) was characterized by one minimum near 218 nm and one maximum near 203 nm, and a negative to positive crossover at 209 nm (Fig. 3). The shape of the spectra shows that frutalin is very similar to jaca-

Table 2. Overall recovery of protein and haemagglutinating activity from *Artocarpus incisa* seeds at various stages of fractionation and purification. The flour was extracted at pH 6.0, centrifuged and the clear supernatant fractionated by dialyses and by treatment with ammonium sulphate to saturation levels as indicated. The fraction $F_{0\,90}$ extract was used for the affinity chromatography on *Adenanthera pavonina* galactomannan. All fractions were recovered after dialysis by freeze drying and tested with rabbit erythrocytes.

Fraction	Amount g	H.U.* (mg^{-1})	Minimum dose† ($\mu g\ ml^{-1}$)	Total H.U. ($\times 10^{-3}$)	Extent of purification (times)
Seed meal	1000.0				
Crude extract	5.25	244	4.10	1280	1
Ppt. 0–60% saturation $F_{0\,60}$	4.30	214	4.67	921	0.88
Ppt. 0–90% saturation $F_{0\,90}$	5.34	221	4.52	1181	0.91
Ppt. 60–90%	0.34	113	8.86	38	0.46
P_{II} (*A. pavonina* column)	1.05	4100	0.24	4300	17.08

* Haemagglutinating unit.

† Minimum dose capable of agglutinating a 2% erythrocyte suspension.

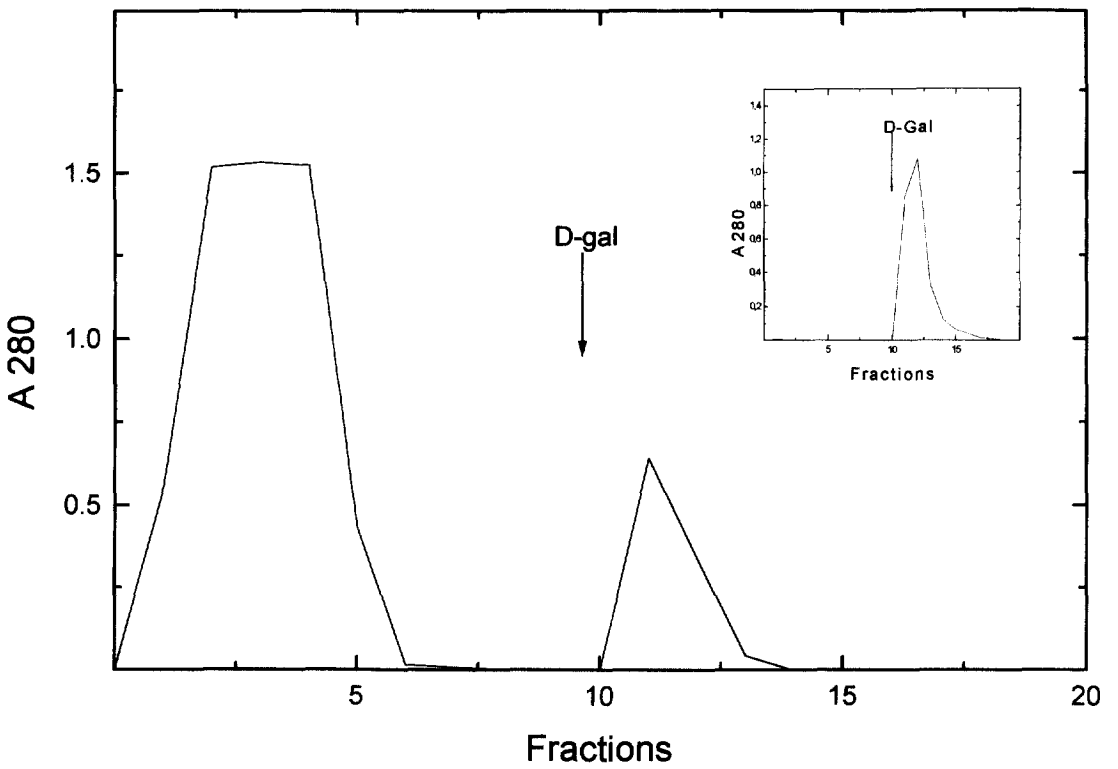

Fig. 1. Purification of the lectin from *Artocarpus incisa*, by affinity chromatography on *Adenanthera pavonina* immobilized galactomannan. The column was equilibrated and first eluted with 0.15 M NaCl to remove the unbound proteins. The lectin was recovered with 0.2 M D-galactose in the equilibrium column. The inset shows the rechromatography of frutalin on agarose-D-galactose column. The column was equilibrated and eluted in the same conditions used for the *A. pavonina* column.

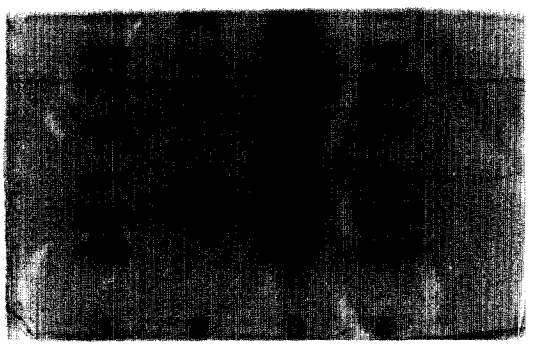

Fig. 2. SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol of the purified lectin from *Artocarpus incisa*. Lanes 1 and 4, standards. Lane 2, frutalin. Lane 3, *Artocarpus incisa* crude extract.

lin, a lectin from *Artocarpus integrifolia* seeds, non-helical and with a three-fold symmetric β-prism fold made up of four-stranded β-sheets [22]. The secondary structure content was estimated using the Convex Constraint Analysis (CCA) program and the following result for the fraction was obtained: α-helix 0%, antiparallel β-sheets 64%, parallel β-sheet and/or β-turn 21%, unordered form 6%, and additional contribution 9%, with an r.m.s. (root mean square) of 1%.

EXPERIMENTAL

Materials. Artocarpus incisa L. seeds were obtained in the State of Ceará (Brazil). Human blood cells from the ABO system were obtained from healthy donors in the Centro de Hemoterapia do Ceará (HEMOCE). Rabbit blood cells were obtained by puncture of the ear's marginal vein of healthy animals. Acrylamide and methylene bisacrylamide were products of Sigma, Superose 12 R, Superdex 75 HR 5/5 and MW, and pI markers were from Pharmacia.

Proximate analysis. Moisture, ash and lipid contents were determined as described in ref. [23], total nitrogen was determined by the method of ref. [24], and total carbohydrate by difference. The protein content was determined from the total nitrogen, using a conversion factor of 6.25.

Lectin extraction. Dehulled *A. incisa* seeds were cut in small pieces and dried in Me_2C. The dried material was then finely ground and stirred with 0.15 M NaCl. The suspensions were left at room temp. for 30 min, filtered in qualitative paper and spun at 10000 *g*, 20 min, 7°. The clear supernatants were used for determining the protein content and haemagglutinating activity.

Lectin purification. The saline crude extract was pptd by $(NH_4)_2SO_4$ (0–90% of saturation), dialysed against 0.15 M NaCl and applied to an *Adenanthera*

Table 3. Amino acid composition of *Moraceae* lectins

	Residues/100		
	Artocarpus incisa	*Artocarpus integrifolia**	*Maclura pomifera**
Asx	8.72	10.68	10.28
Thr	7.38	7.83	8.01
Ser	7.02	8.75	7.79
Glx	8.64	5.51	7.52
Pro	5.84	4.50	4.13
Gly	12.35	12.62	13.21
Ala	4.86	2.34	2.21
Cys	nd†	nd†	nd†
Val	5.69	7.86	8.74
Met	1.17	1.17	0.58
Ile	6.87	6.28	7.86
Leu	6.24	7.01	5.87
Tyr	5.80	8.36	7.05
Phe	7.34	7.66	6.50
Lys	6.71	6.25	5.48
His	1.65	0.89	0.78
Arg	2.35	1.47	2.82
Trp	1.38	0.81	1.16

* ref. [5].
*† Not detected.

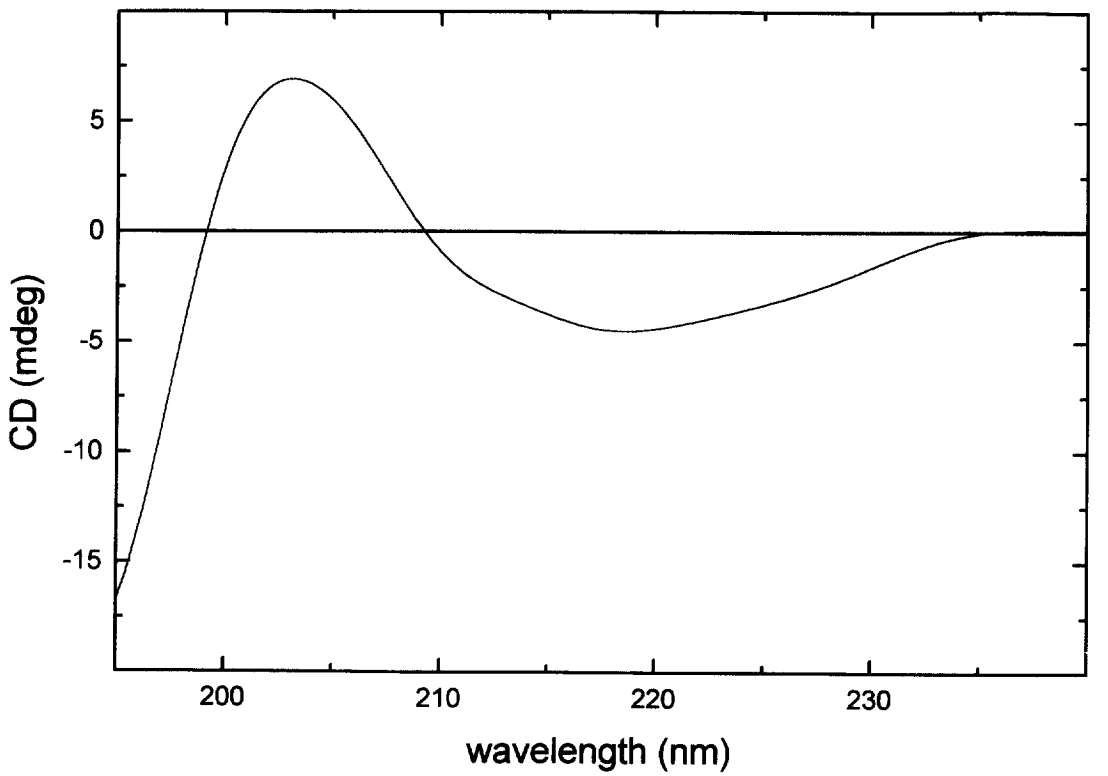

Fig. 3. CD spectra of frutalin (150 μg ml^{-1}) lectin in PBS, pH 7.4, at 25 .

pavonina cross-linked galactomannan column [25] equilibrated with the same soln. After removing the unbound material, the lectin was desorbed from the column with 0.2 M D-galactose in the equilibrium soln. For characterizing the purity of the lectin, the peak obtained in the affinity chromatography on

Adenanthera pavonina column was rechromatographed on a D-galactose-agarose column, in the same conditions.

Protein concn. This was determined by the method described in ref. [26], using bovine serum albumin (BSA) as standard. Readings at 280 nm were also used to determine protein content of the column eluates.

Carbohydrate content. The neutral sugar content of the isolated protein was estimated by the phenol–sulphuric acid method of ref. [21] using glucose as the standard.

Haemagglutination activity. Clumping of red blood cells by the various frs obtained during purification was estimated as described before [27], in small glass tubes where a series of 1:2 dilutions of the various frs obtained during purification, in 0.15 M NaCl, were mixed with 0.25 ml of a 2% suspension of erythrocytes. The degree of agglutination was monitored visually after the tubes had been left at 37° for 30 min and subsequently left at room temp. for a further 30 min. One H.U. was defined as the reciprocal of the highest dilution still giving a visible agglutination. Sp. act. was expressed as Haemagglutination Units (H.U.) mg^{-1} or as the minimum concn of protein ($\mu g\ ml^{-1}$) still giving activity.

Sugar specificity. The carbohydrate-binding specificity of the lectin was estimated by the ability of a series of simple sugars to inhibit the haemagglutination of rabbit erythrocytes. Two-fold dilutions of sugar (1 M initial concn) solns in 0.15 M NaCl to a final vol. of 0.25 ml were mixed with 0.25 ml of the lectin soln (1 mg ml^{-1}), and allowed to react for 37 min at 30°. An erythrocyte suspension (0.25 ml) was then added and the mixt. left for 30 min at 37° and 30 min at room temp. The haemagglutinating titles obtained were compared with a non-sugar containing blank. In this study the following sugars were used: D(+) xilose, D(−) arabinose, D(−) fructose, D(+) galactose, D(+) glucose, D(+) mannose, L(+) rhamnose, glucuronic acid, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, D(−)celobiose, D(+) lactose, D(+) maltose, D(+) trehalose, D(+) melezitose, raffinose; and the glycoproteins: apotransferrin, asialofetuin, fetuin and tiroglobulin.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). This was carried out on vertical 2 mm gel slabs of 17.6% polyacrylamide sepn gel and 4% stacking gel run at 13 mA for 4 hr [28]. Samples were dissolved in 0.01 M Na-Pi pH 7.0, 2% SDS buffer with 1% 2-mercaptoethanol and incubated at 100° for 15 min. A few crystals of sucrose were dissolved in the samples which were then applied to the gel. The electrophoresis was conducted at a constant current of 13 mA for 4 hr. The protein bands were visualized by staining with Coomassie Brilliant Blue R-250. Estimation of M_r of the lectin sub-units was made using bovine serum albumin (66 k), egg albumin (45 k), carbonic anhydrase (30 k), soybean trypsin inhibitor (20.1 k), cytochrome C (12.4 k) and kalikrein inhibitor (6.6 k).

Analytical isoelectric focusing. This was performed on precast polyacrylamide gels (FastGel, Pharmacia, Sweden) in the pH-range of 3–9. The pH gradient in these gels was determined from the results of simultaneous runs performed with a wide-range isoelectric-focusing protein calibration kit (Pharmalyte 3–10). The stained gels were traced onto graph paper and the position of the bands plotted *vs* the known isoelectric point of the proteins in the calibration kit.

Amino acid composition. Amino acid analysis was performed after hydrolysis at 110° of lectin samples (in sealed glass tubes under N_2) for 20 hr in 6 M HCl. After hydrolysis, HCl was removed by evaporation and the residue was analysed, in a BIOCHROM 20 (Pharmacia) amino acid analyser. Tryptophane was determined spectrophotometrically, as described in ref. [29].

Effect of EDTA and Ca^{2+} and Mn^{2+}. The purified lectin (2 mg) was dissolved in and dialysed exhaustively (48 hr) against 0.2 M EDTA, followed by dialysis against 0.15 M NaCl (24 hr). The haemagglutinating activity was determined in the soln before and after the addition of $CaCl_2$ and $MnCl_2$ up to 10 mM.

Circular Dichroism (CD) measurements and estimation of secondary structure. CD spectra were recorded using a Jasco J-720 spectropolarimeter over wavelength range 195–240 nm. Measurements were made on frutalin samples of protein concn in 0.15 mg ml^{-1}, in quartz cuvettes of 1 mm path length. Spectra were typically recorded as an average of 8 scans and measured in PBS pH 7.4. CD spectra were obtained in m degree ellipticity (θ) scale and transformed using the mean wt residue and concn prior to the secondary structure analysis. Analysis of the CD spectra in terms of the secondary structure content was performed using the convex constraint analysis (CCA), developed by Perczel and Fasman and based in the algorithm simplex. This method allows the deduction of the spectral contribution of the common secondary structures (deconvolution) through the direct use of the experimental CD curves of proteins. Spectra of 25 proteins were used as the base for deduction of the spectral contribution of secondary structures [30], [31].

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