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Purification of a Glucose/Mannose Specific Lectin, Isoform 1, from Seeds of *Cratylia mollis* Mart. (Camaratu Bean)

MARIA T. S. CORREIA AND LUANA C. B. B. COELHO*

Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Pernambuco, UFPE, 50670-420, PE, Brazil

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ABSTRACT

A quantitatively main molecular form of *Cratylia mollis* lectin, isoform 1 (iso 1) was purified by affinity chromatography on Sephadex G-75, followed by ion exchange chromatography on CM-cellulose. Another lectin form was identified in the latter step. Iso 1 is specific for glucose/mannose, with a main subunit of 31 kDa mol wt; the native protein is basic (pI 8.5–8.6) and the constituent polypeptides had a pI range of 5.15–7.75. An antibody to the protein was raised in a rabbit, and the conjugate was active in an immunosorbent assay.

Index Entries: Lectin; isoform; lectin purification; antilectin IgG; antilectin IgG-peroxidase.

INTRODUCTION

More than a hundred lectins, bifunctional proteins able to choose carbohydrates or other ligands (*1–3*), have been purified with different recognition specificities, resulting in a vast use of these molecules as a powerful tool in medical and biological research (*4–7*).

*Author to whom all correspondence and reprint requests should be addressed.

Plants constitute a material of excellence to isolate lectins and the greater majority of these proteins were obtained and characterized from seeds (*8–10*). FABACEAE seed lectins, with glucose/mannose specificity, in general, are purified by an affinity chromatography step in Sephadex and are nonspecific to human ABO blood group determinants (*11,12*). Structurally, they can be divided in two groups: with one subunit and with two distinct subunits (*12*). Different biological activities have been detected to glucose/mannose lectins (*13*), even if they present a high homology in the primary structure (*14*) and antiserum recognition identity (*15*).

Two minor molecular forms of a lectin were purified and partially characterized from seeds of *Cratylia mollis* (*16*), camaratu bean. The legume is a native forage from the semiarid region of Pernambuco, state in the Northeast of Brazil; the plant belongs to the tribe *Phaseoleae*, subtribe *Dioclinae*, which contains the genus *Canavalia*, botanically related to *Cratylia*. The first lectin to be purified was concanavalin A (Con A), from *Canavalia ensiformis;* this lectin is one of the most broadly evaluated in its characteristics and applications (*17–19*).

In this work, we describe the purification to homogeneity of the main lectin, quantitatively, from seeds of C. *mollis*, isoform 1, iso 1. The development of its antiserum, as well as the purification and conjugation of IgG to peroxidase are also presented.

MATERIALS AND METHODS

Hemagglutinating Activity

Fresh erythrocytes from human (ABO), chicken, duck, quail, rabbit, and steer were obtained according to Bukantz et al. (*20*). Rabbit cells were also fixed in glutaraldehyde by the method of Bing et al. (*21*). Samples of the lectin (50 μL) were twofold serially diluted in 0.15*M* NaCl, in microtiter plates. An erythrocyte suspension (2.5% v/v in 0.15*M* NaCl, 50 μL) was added and the titer was read after 45 min. The hemagglutinating activity (HA) is defined as inverse of the last dilution presenting hemagglutination, and the specific HA (SHA) corresponded to the HA divided by the protein concentration. The erythrocyte suspension used for general HA evaluation was prepared with glutaraldehyde-treated rabbit erythrocytes.

HA in the presence of ions was measured by twofold serially diluted lectin fractions in Ca^{2+}, Mg^{2+}, and Mn^{2+} solutions (0.3–20.0 m*M*) contained in 0.15*M* NaCl and also, the fractions were incubated with the same ions, 10 m*M* in 0.15*M* NaCl, as well as dialyzed with EDTA 10 and 100 m*M*, according to Shiomi et al. (*22*).

HA inhibition (HAI) was assayed by a twofold serially diluted sample (50 μL) of iso1 (100 μg/mL) in 50 μL of carbohydrate solutions, followed by a 45-min incubation and addition of the erythrocyte suspension. The carbo-

hydrate concentration varied from 0.4–400 m*M* and the HAI is the concentration of inhibition required to inhibit 4 HA units. L-Arabinose, D-fructose, D-galactose, D-glucose, D-lactose, maltose, D-mannose, L-rhamnose, D-ribose, sucrose, and xylose were from Merck (Darmstadt, Germany); all the other carbohydrates were from Sigma (St. Louis, MO).

Lectin Purification

C. mollis seeds were collected in Ibimirim City, State of Pernambuco and botanical identification was kindly performed by Marcelo Ataide, from the Agriculture and Cattle Raising Research Company of Pernambuco (IPA). An ether extract of a seed flour was performed previously (*23*). Whole seeds were washed with distilled water, dried at room temperature (rt), and blended in 0.15*M* NaCl (10% w/v). After 16 h of gently stirring at 4°C or rt, the extract was filtered through a cheesecloth and centrifuged for 12,100*g* (15 min, at 4°C).

Fraction 1, F-1 (extract at 4°C), was ammonium sulfate fractioned (F0–40 and F40–60). The F40–60 dialyzed in 0.15*M* NaCl (F-2) was affinity chromatographed on Sephadex G-75 (Sigma) in a column (70.0 × 1.9 cm) containing 200 mL of packed matrix, equilibrated with 0.15*M* NaCl. After sample application, 0.15*M* NaCl was passed through the column until the $A_{280\ nm}$ was less than 0.1. Elution was performed with 0.3*M* D-glucose in 0.15*M* NaCl. Fractions with the highest $A_{280\ nm}$ were pooled and exhaustively dialyzed in 10 m*M* citrate phosphate buffer, pH 5.5 (F-3). The latter preparation was chromatographed in a column (31.0 × 1.5 cm) containing 50 mL of packed CM-cellulose (Sigma) equilibrated with the citrate phosphate buffer. The column was eluted with a 0–0.4*M* NaCl linear gradient; the highest HA peak obtained (F-4, iso 1) was chromatographed in a Bio-Gel P-2 column (8.0 × 1.8 cm) containing 20 mL of packed matrix. The latter column was previously equilibrated in the citrate phosphate buffer and was eluted with the same buffer (F-5).

Heat Treatment of Fractions

F-1, F-3, and F-4 (100 μg of protein/mL in 0.15*M* NaCl) were heated according to Shiomi et al. (*22*); F-5 (150 μg of protein/mL) was heated for 4 h at 60°C (three times) and 80°C (one time). After heating, the solution was cooled in an ice bath, kept at −20°C for 16 h, and the HA was measured.

Electrophoresis and Isoelectric Focusing in Polyacrylamide Gel

Native proteins were electrophoresed and isoelectric focused according to Reisfeld et al. (*24*) and LKB (*25*), respectively. Denatured and reduced samples were resolved according to Laemmli (*26*) and submitted

to protein (*26*) and carbohydrate stainings (*27–28*). The pI pattern of the polypeptides was evaluated (*29*) using ampholines with pH ranges of 4.0–6.0, 6.0–8.0, and 3.5–10.0 (LKB, Bromma, Sweden).

Preparation and Evaluation of Anti-Iso 1 Serum and IgG

F-5 (150 μg of protein) in 1 mL of 0.15*M* sodium phosphate buffer, pH 6.2, containing 0.15*M* NaCl, was emulsified with 1 mL of Freund's complete adjuvant (first inoculation) or 1 mL of Freund's incomplete adjuvant (seven following inoculations) and intradermally injected into a male, New Zealand white rabbit, in a fortnight interval. Immediately before each inoculation, 10 mL of blood were collected from the ear central artery. The blood was kept in a glass tube at rt for 1 h, and then stored at 4°C for 2 h. The serum obtained was centrifuged at 1300*g*, for 5 min, at rt. Aliquots were stored at −20°C. The antilectin serum (3 mL) was chromatographed in a column (6.5 × 1.0 cm) containing 4.5 mL of protein A-Sepharose CL-4B (Sigma) based on Hudson and Hay (*30*). Antilectin serum and IgG were evaluated by double immunodiffusion (*31*): in a 1% (w/v) agarose gel in 0.15*M* NaCl, containing 0.1*M* methyl-α-D-mannoside.

Conjugation of Anti-Iso 1 IgG to Peroxidase and Its Evaluation with a Lectin Immunosorbent Assay (LIA)

Anti-iso 1 IgG (3 mg/mL), dialyzed against 0.1*M* sodium phosphate buffer, pH 6.8, was conjugated to peroxidase (Merck) based on Weir (*32*).

A lectin immunosorbent assay (LIA) was performed with lectin (5 μg/mL) in 0.1*M* $NaHCO_3$, containing 0.5*M* NaCl (50 μL) and was adsorbed to a microtiter plate for 16 h at 4°C. The plate was washed three times with Dulbecco's PBS (-) Nissei (Tokyo, Japan), containing $CaCl_2$ (0.1 mg/L), $MgCl_2$ (0.1 mg/L), and 0.1% (v/v) Tween 20 (Merck), buffer A. Incubation (2 h at 4°C) with 50 μL of anti-iso 1 IgG double diluted (2^{-1}–2048^{-1}) or not, or with 50 μL of anti-iso 1 IgG-peroxidase diluted (8^{-1}–2048^{-1}) in buffer B (buffer A containing 0.5% w/v casein, Merck, and 0.1*M* methylα-D-mannoside), was followed by three washes with buffer A. Another incubation with 50 μL of sheep-rabbit IgG-peroxidase (Serotec, Oxford, UK), diluted in buffer B (1000^{-1}) was performed, for 2 h at 4°C, when anti-iso 1 IgG was used. Following, washes were performed as described. Then, a fresh solution (50 μL) of 0.08% (w/v) dihydrochloride *o*-phenylenediamine (74 mg/100 mL, Nakara, Japan) in 10 m*M* citrate phosphate buffer, pH 6.0, containing H_2O_2 20 vol (30 μL/100 mL), was added. The mixture was kept in the dark for 30 min; the reaction was stopped with 100 μL of 0.5*N* citric acid and read at 492 nm in a spectrophotometer (EIA Reader, 2550, Bio-Rad, Hercules, CA).

Table 1
Purification of Iso 1[a]

Sample, fraction	Volume, mL	Protein, mg/mL	Titer	Specific activity, protein/titer	Total activity, titer × volume × 10^3	Yield, %
1	754.0	6.6	8192	1241	6177	(100)
2	39.0	45.0	262144	5825	10224	165[b]
3	54.0	9.6	131072	13653	7078	69[c]
4	18.0	0.4	2048	5120	147	37[c]
5	3.3	0.2	2048	10240	7	82[c]

[a]The lectin activity was measured with glutaraldhyde-treated rabbit erythrocytes.
[b]Percentage of total activity from the crude extract.
[c]Total activity recovered × 100/Total activity chromatographed.

RESULTS

Lectin Purification

C. mollis seed flour showed an ether extract with a low value of 2.1%; no defating step was included to the purification protocol.

The SHA of 19.3 (4 h) and 1241 (16 h) justified a longer period of lectin extraction. F-1 was ammonium sulfate fractionated, and 87% of lectin activity was obtained in F-2, which had a yield of 165% in relation to F-1 (Table 1). F-2 was affinity chromatographed and the native protein of F-3 (peak II, Fig. 1) was resolved into two basic protein bands (Fig. 2A). Protein elution with methyl-α-D-mannoside (0.05, 0.15, and 0.3*M*), D-mannose (0.3*M*), or sucrose (0.3*M*) resulted in chromatographic and electrophoretic patterns similar to F-3. Rechromatography of F-3 on Sephadex G-75 resulted in total protein adsorption to the matrix but no further purification of the glucose desorbed material. F-3 was then chromatographed in CM-cellulose and the protein peaks obtained, I and II, had HA (Fig. 3). Peak I, iso 4, gave an electrophoretic migration distinct of peak II, iso 1 (Fig. 2B,C), as basic and native proteins. F-3, which contained iso 1 and 4 or F-4 (iso 1) were resolved in a native molecular weight of 50 kDa, by FPLC (results not shown).

HA and Inhibition Evaluation of Iso 1 Fractions

The HA of iso 1 fractions were evaluated with distinct erythrocytes and the highest HA were obtained with quail and rabbit cells (Table 2). HA of the preparations were not altered in the presence of ions; F-1 and F-3 did not change activities after dialysis against EDTA.

F-1, F-3, and F-4 were stable when heated to 80°C, but lost activity at 90°C. F-5 was active (HA = 2048) after successive thermic treatments; at

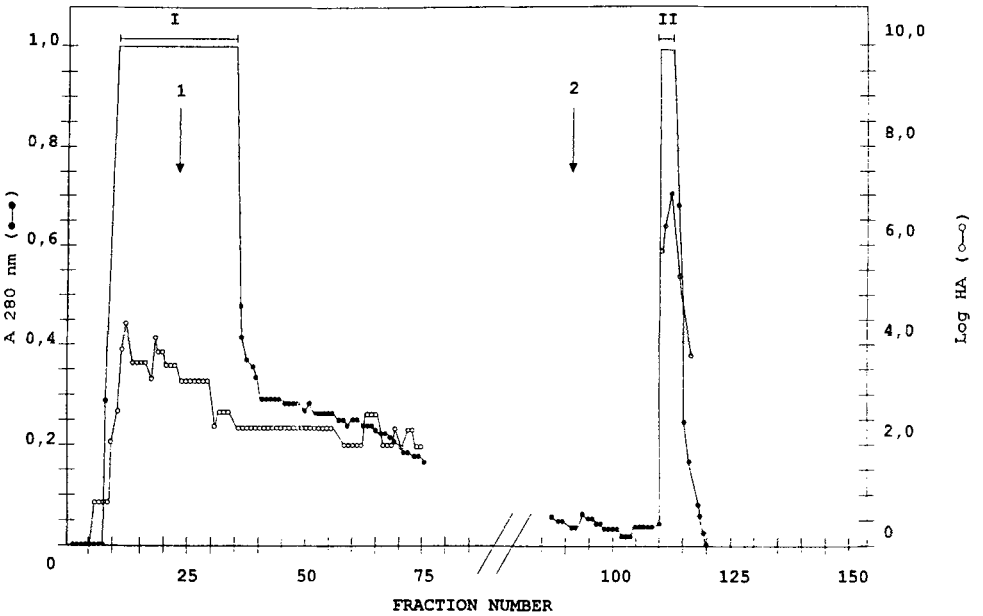

Fig. 1. Elution pattern of F-2 on Sephadex G-75 affinity column. A sample (1500 mg of protein) was applied in a flowrate of 36 mL/h and 10 mL fractions were collected. The arrows indicate the elution with 0.15*M* NaCl (1) and 0.3*M* glucose in 0.15*M* NaCl (2). HA was followed with glutaraldehyde-treated rabbit erythrocytes. Peak I. Unadsorbed protein. Peak II. F-3.

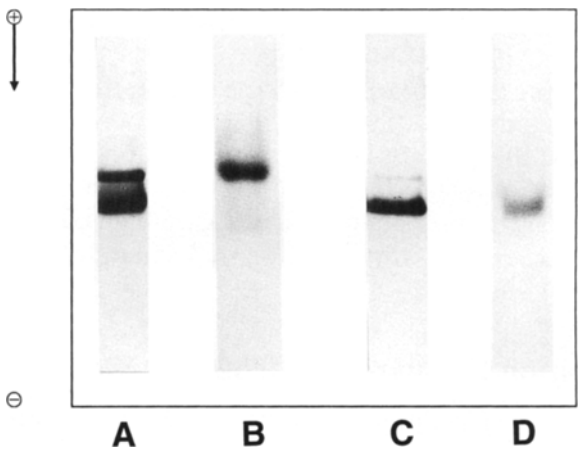

Fig. 2. Polyacrylamide gel electrophoresis to basic and native proteins, pH 4.3. Samples of F-3 (**A**), peak I of CM-cellulose, iso 4 (**B**), peak II of CM-cellulose, iso 1 (**C**) and F-5 (**D**), containing 200, 200, 200, and 100 μg of protein, respectively. Protein was stained with Amido black according to Reisfield (24).

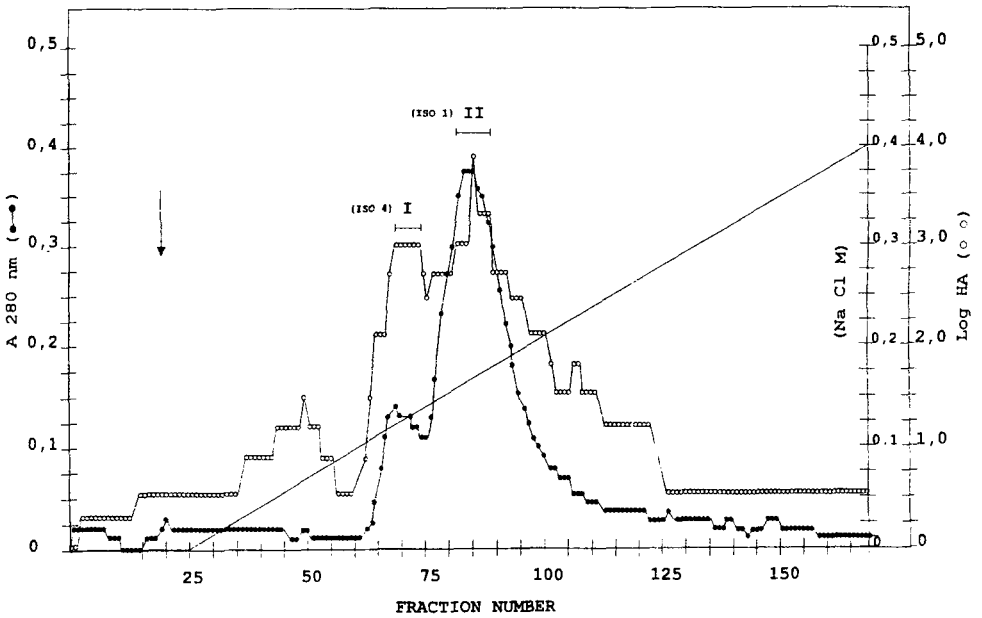

Fig. 3. Elution pattern of F-3 on a CM-cellulose column. A sample (30 mg of protein) was applied in a flowrate of 10 mL/h and 2 mL fractions were collected. The arrow indicates where elution with the linear gradient was started. HA was followed with glutaraldehyde-treated rabbit erythrocytes.

Table 2
Specific Hemagglutinating Activity Assayed with Erythrocytes of Different Animals[a]

	SHA[b]				
Erythrocytes	F-1[c]	F-2	F-3	F-4	F-5
Human					
A	77	45	106	40	160
B	2.4	0.7	—[d]	—	—
O	115	728	426	320	320
AB	9.7	2.8	—	—	—
Chicken	9.7	2.8	—	—	—
Duck	77	91	—	—	—
Quail	9929	10984	10922	81920	163840
Rabbit	1241	5825	13653	5120	10240
Steer	0.3	—	—	—	—

[a]Fresh erythrocytes were used in a 2.5% (v/v) suspension in 0.15*M* NaCl.
[b]SHA, specific hemagglutination activity.
[c]F, fraction.
[d](—), undetected SHA.

Table 3
Hemagglutinating Activity Inhibition of Iso 1[a]

Carbohydrate[b]	Concentration, $M \times 10^{-3}$
Methyl-α-D-mannoside	0.4
Trehalose	3.1
Methyl-α-D-glucopyranoside	3.1
D-Mannose	6.2
D-Fructose	6.2
D-Glucose	12.5
L-Rhamnose	25
N-Acetyl-D-glucosamine	50
Sucrose	50
D-Xylose	100
L-Fucose	400

[a]The inhibition was evaluated by the minimal concentration of carbohydrate required to inhibit 4 HA units of iso 1 (100 μg/mL).

[b]Raffinose, 1-*O*-methyl-α-D-galactopyranoside, melibiose, 1-*O*-methyl-β-arabinopyranoside, *N*-acetyl-D-galactosamine, D-fucose, D-galactose, D-ribose, 2-deoxy-D-galactose, 2-acetamido-1,amine-1,2-deoxy-D-glucopyranoside, *N*-acetyl-β-D-mannosamine, lactose, L-arabinose, and starch, were not inhibitors.

80°C the latter prep showed a turbidity, but the activity was maintained. Iso 1's fractions were active for 1–3 yr at −20°C, even after at least 10 cycles of freezing and thawing. When turbidity was observed, samples were centrifuged with no loss of activity.

Iso 1 is a glucose/mannose lectin, with methyl-α-D-mannoside as the best carbohydrate inhibitor and, 10 of 25 tested carbohydrates inhibited the activity (Table 3).

Electrophoresis and Isoelectric Focusing in Polyacrylamide Gel

Electrophoresis under denaturing and reducing conditions resolved F-3, F-4, and F-5 with a similar pattern, in the absence or presence of β-mercaptoethanol: a main band of 31 kDa and two very faint bands of 28 and 16 kDa (Fig. 4). The carbohydrate stainings used were negative to iso 1 polypeptides. Native iso 1 (F-4) was resolved in a diffuse protein band, with pI values of 8.5–8.6 by native isoelectric focusing. F-4 polypeptides had pI values (Fig. 5) of 5.15, 5.95, 6.10, and 7.75 (main bands), as well as pI values of 6.00 and 6.05 (faint bands); however, F-5 showed polypeptides with pI values of 5.35, 5.55, 6.00, 6.35, 6.55, 6.80, and 7.75 (main bands), as well as pI of 5.70, 5.85, 6.45, 6.65, 6.70, 6.75, 6.90, and 6.95 (very faint bands).

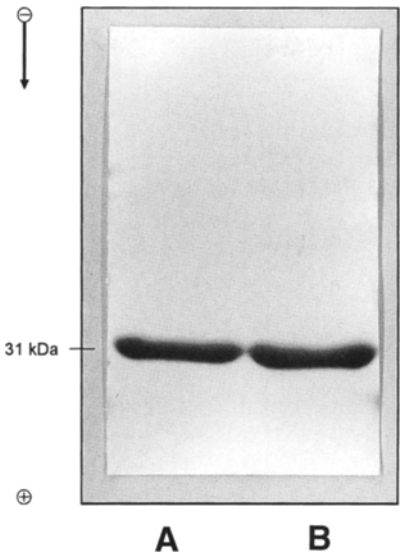

Fig. 4. SDS-polyacrylamide gel electrophoresis of F-4. Samples containing 50 μg of protein were denatured and reduced with β-mercaptoethanol (**A**) or nonreduced (**B**). Protein was stained with Coomassie brilliant blue, according to Laemmli (*26*).

Double Immunodiffusion and LIA

Iso 1 antigenicity was evaluated after inhibition with methyl-α-D-mannoside (0.3), since the lectin precipitated proteins from nonimmune serum. Immunodiffusion with anti-iso 1 serum and anti-iso 1 IgG resulted in an unique immunoprecipitation line to F-4 and F-5 (Fig. 6). The duplicity of lines in F-1 and F-2 could be attributed to the presence of distinct *C. mollis* isoforms. LIA was positive with an absorption of 0.112 to iso 1, when anti-iso 1 IgG was diluted 256 times; an absorption of 0.125 was obtained to anti-iso 1 IgG-peroxidase, 16 times diluted.

DISCUSSION

Extraction and salt fractionation are important steps in protein purification, since activity can change considerably in distinct protocols. The presence of an inhibitor of protein or carbohydrate nature has been suggested in lectin purification steps (*33,34*), to justify the high yields obtained after extraction or salt precipitation, as obtained to iso 1 fractions.

The stability of proteins to temperature, the free energy of unfolding, is dependent on the hydrophobic effect and the conformational entropy (*35*). Seed lectins are stable even after months or years kept frozen, or successive freezing and thawing (*34,36,37*). The thermal stability of lectins

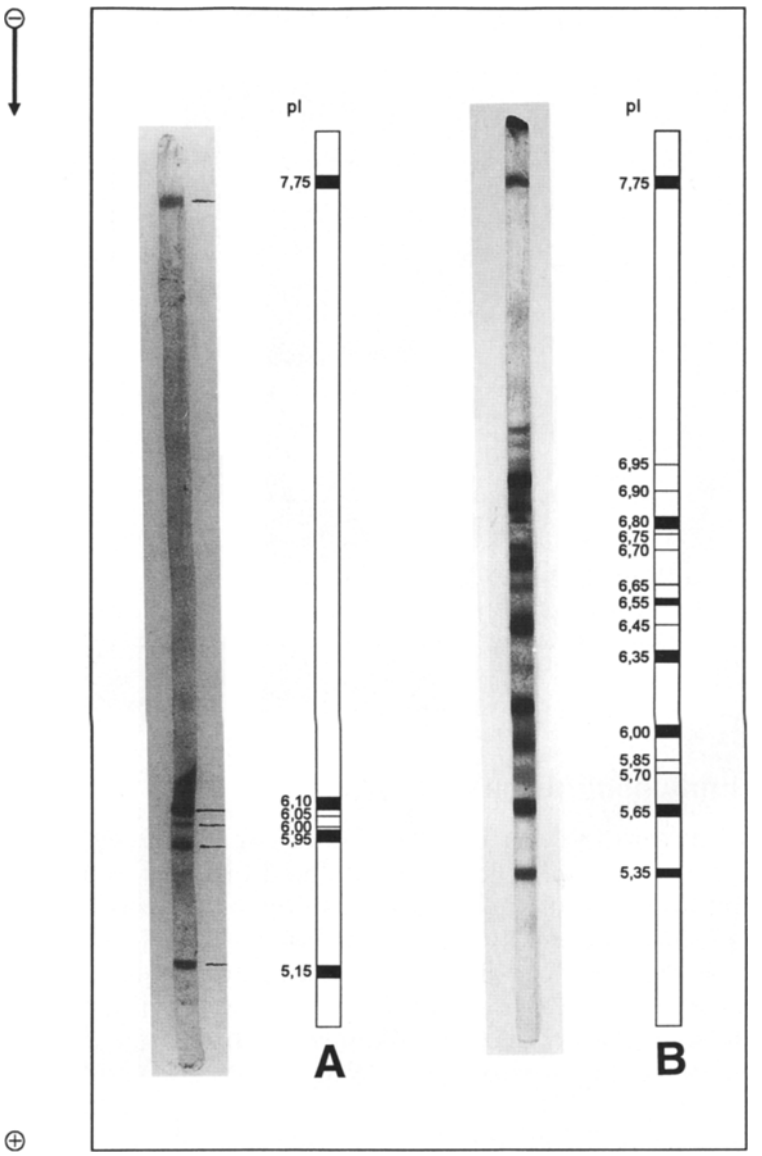

Fig. 5. Isoelectric focusing of iso 1 fractions in polyacrylamide gel. Samples contained 100 μg of protein of F-4 (**A**) and F-5 (**B**). Protein was stained with Coomassie brilliant blue according to O'Farrell (*29*).

from distintic sources is variable, however, several of these proteins only lose activity above 60°C (*22,36,38–41*).

The higher number of bands observed to iso 1, F-5, could result from a further desaggregation of polypeptides, noncovalently bound, resulting from the protein fragility, since it was submitted to another chromatographic step. It is known the ability of urea to disrupt protein aggregates and reduce proteins to their constituent polypeptides (*42*).

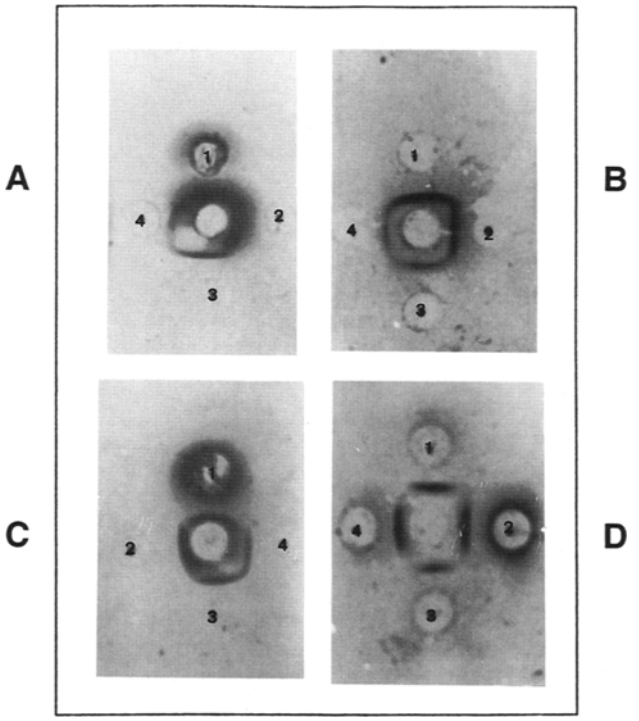

Fig. 6. Double immunodiffusion on agarose gel. To the central wells 10 μL of anti-iso 1 serum (**A,B**) and anti-iso 1 IgG (**C,D**) were added. Wells 1, 2, 3, and 4, contained 10 μL of F-2, F-3, F-4, and F-5, after thermic treatment (**A,C**) and F-5, without and with dilutions of 2^{-1}, 4^{-1}, and 8^{-1} (**B,D**), respectively.

Among the carbohydrate inhibitors to iso 1 only rhamnose differs in position C-4. Lectins support a variation in positions C-2 and C-3 from monosaccharides and derivatives, but do not accept a variation in position C-4 (*3*). Methyl-α-D-mannoside, D-mannose, as well as L-rhamnose, present neighbor hydroxils. The latter carbohydrate was the inhibitor to *Panstrongylus megistus* lectin, a result attributed to the ability of neighbor hydroxils to promote the best interaction in the lectin site (*43*). The definition of lectin specificity is important to establish the affinity matrix to be used in the protein purification (*44*) and to inhibit the lectin function domain (*45,46*). In some assays the binding sites must be carbohydrate blocked (*31*).

Immunosorbent assays could be performed indirectly, by using anti-lectin IgG, followed by a secondary antibody conjugated to peroxidase (*47*), with the advantage to amplify the interaction; or directly with anti-lectin IgG conjugated to peroxidase. In the first situation the secondary antibody may give a nonspecific interaction.

Multiple molecular forms constitute a characteristic of *C. mollis* lectin: iso 1, quantitatively, the main lectin, iso 2 and 3 (*1*) as well as iso 4. Iso 1, 2, and 3 were so-called according to the distinct electrophoretic migration

in polyacrylamide gel to native and basic proteins. Iso 2 had the same migration as native and basic iso 4. A main polypeptide of 31 kDa was present in iso 1, iso 3 (glycosylated, distinctly from Con A, a subtribe *Dioclinae* lectin) and iso 4; iso 2 had a 60 kDa polypeptide. Isoenzymes, determined by multiple gene loci, presented different physiological functions (*48*). Variation in the composition of carbohydrate side-chains in multiple forms of enzyme suggest differences of physiological significance (*49*). Also, multiple forms of enzymes, which arise by posttranslation modifications of a single gene product, may differ or not (*50*) in their catalytic characteristics. *C. mollis* seed isoforms (*16*) or isolectins, among other applications, have been used to explore cell surfaces, to evaluate homologies among lectins of the same or distinct species and, as an affinity matrix, to purify glycoproteins.

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