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PURIFICATION OF HUMAN ERYTHROCYTES SPECIFIC LECTINS FROM RICE BEAN, *PHASEOLUS CALCARATUS* SYN. *VIGNA UMBELLATA*, BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Two lectins, an N-acetylgalactosamine-binding lectin, lectin-I, which reacts specifically with human erythrocytes of blood group A, and a galactose-binding lectin, lectin-II, which is specific for human blood group B erythrocytes, have been isolated and purified from rice bean, *Phaseolus calcaratus* syn. *Vigna umbellata*, by a salt solubility pH-dependent method, chromatofocusing and high-performance liquid chromatography. The homogeneity of the lectins was determined by liquid chromatography and polyacrylamide gel electrophoresis. The purified lectin-I of molecular mass 80 000 is possibly composed of two subunits of molecular mass ca. 18 000 and 22 000, respectively, whereas lectin-II of molecular mass 100 000 appears to be composed of a monomeric protein of molecular mass 25 000. One endogenous lectin-binding protein was also isolated and purified by liquid chromatography. The endogenous lectin-binding protein of molecular mass 40 000 affects the activity of the A-group specific lectin more than that of the B-group specific lectin. The endogenous lectin-binding protein appears to be composed of a monomeric protein of molecular mass 20 000.

INTRODUCTION

The seeds of *Phaseolus* spp. contain significant amounts of lectins, e.g. red kidney bean, wax bean, black bean, lima bean, tora bean and others [1–5]. Lectin from *Phaseolus* species can generally be subdivided into the following groups: non-specific; blood-group specific; toxic hemagglutinin [6]. Seeds with no hemagglutinating activity have also been reported [4]. *Phaseolus lunatus* syn. *limensis* lectin exhibits A erythrocyte specificity [1]. Little attention has been given to the hemagglutinating characteristics of the seed extracts of other *Phaseolus*

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spp. Toms [7] reported that the hemagglutinating reactions of *P. trilobus* Ait. (L.), *P. leucanthus* Piper and *P. polystachios* (L.) BSP have potential value in ABO blood grouping; however, lectins from these sources have not been isolated and characterized.

Rice bean, *P. calcaratus* syn. *Vigna umbellata*, like other pulse crops, is grown both for food and for fodder [8]. Schertz et al. [9] noted that the extract of *P. calcaratus* seed has human blood group specific erythroagglutinating properties. This paper describes a purification procedure and partial characterization for *P. calcaratus* lectins by conventional techniques with high-performance liquid chromatography (HPLC) as a final step. In addition, the endogenous lectin receptor has also been observed.

EXPERIMENTAL

Reagents and chemicals

Rice bean, *P. calcaratus*, was purchased from Bidhan Chandra Krishi University (Kalyani, India). The chromatofocusing gel PBE-94 was from Pharmacia (Uppsala, Sweden). HPLC-grade methanol was from Spectrochem (Bombay, India). Normal typed human erythrocytes were obtained from the Medical College Blood Bank (Calcutta, India). Blood from different species of animals used in this study was procured from the Institute Animal House. Alcohol dehydrogenase, yeast (M_r 150 000), concanavalin A (M_r 100 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 29 000), cytochrome C (M_r 12 000), MW-SDS-70 molecular mass marker kit, glucose, N-acetyl-D-glucosamine (GlcNAc), galactose, N-acetyl-D-galactosamine (GalNAc), mannose, arabinose, fucose, xylose and sialic acid were all from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade.

Lectin purification

Step 1. Dehusked seeds (15 g) were soaked in 250 ml of 0.15 *M* phosphate-buffered saline (pH 7.2) containing 10 m*M* calcium chloride, 10 m*M* magnesium chloride and 10 m*M* manganese chloride for 12 h at 4 °C, and homogenized. The homogenate was filtered through cheese cloth. The supernatant solution (crude extract) was collected after centrifugation for 30 min at 15 000 *g*. The crude extract was dialysed overnight against McIlvaine buffer (pH 4.8) in 0.2 *M* sodium chloride and then centrifuged at 25 000 *g* for 20 min. The precipitate was dissolved in 0.1 *M* phosphate buffer (pH 7.2) containing 0.20% sodium azide in 0.75 *M* sodium chloride at 25 °C. The solution was dialysed and centrifuged as above. The precipitate obtained on centrifugation was called globulin fraction 1 (GF-1). The supernatant obtained at this stage was pooled with the supernatant obtained in the previous step. The combined supernatant was dialysed against deionized water with several changes for 24 h and was centrifuged at 25 000 *g* for 20 min. This precipitate was called globulin fraction 2 (GF-2). The supernatant at this stage still contained protein and was called albumin proteins. The GF-1 and GF-2 preparations were dissolved in 0.2 *M* sodium chloride in 0.15 *M* phosphate buffer (pH 7.5).

Step 2. The chromatofocusing analysis of GF-1 fraction was carried out on the PBE-94 column of 25 ml packed volume. The column was repeatedly washed and equilibrated with 0.025 *M* sodium acetate–acetic acid buffer (pH 5.0). The GF-1 solution in 0.15 *M* phosphate buffer (pH 7.5) containing 0.2 *M* sodium chloride was dialysed against 0.025 *M* sodium acetate–acetic acid buffer (pH 5.0) containing 0.15 *M* sodium chloride with several changes. An aliquot containing 6 mg of protein was loaded on the chromatofocusing column. Protein fractions were eluted from the column with a pH gradient of 5.0–3.0 using 0.025 *M* glycine–HCl buffer (pH 3.0) at a flow-rate of 5 ml/h. The proteins, eluted at pH 4.7 and pH 3.5 from the column, possessed erythroagglutinating activity. The protein eluted at pH 4.7 agglutinated B-group erythrocytes (lectin-II). The protein eluted at pH 3.5 agglutinated A-group erythrocytes (lectin-I).

Step 3. HPLC assays were conducted using the Waters Assoc. Model 440 system (Milford, MA, U.S.A.). A protein PAK I 125 column was used. The results were recorded on a Houston Instruments Omniscribe recorder (10 mV full scale) attached to the HPLC system. The column was washed with methanol and subsequently with water. The HPLC assay was carried out in 0.15 *M* phosphate-buffered saline (pH 7.2) containing 10 m*M* calcium chloride, 10 m*M* magnesium chloride and 10 m*M* manganese chloride. All solvents were filtered (0.45 μm) and deaerated by sonication prior to use. The individual peaks obtained from chromatofocusing were filtered through a Millipore filter of pore size 0.45 μm, and 50–100 μl sample volumes were injected into the loop. The column effluent, at a flow-rate of 0.7 ml/min, was monitored at 280 nm using 0.1 (0.02–2.0) a.u.f.s. The eluted peaks were collected.

Molecular mass determination

The molecular mass of the purified lectins, lectin-I and lectin-II, and the endogenous lectin inhibitor were estimated [10] by gel permeation on a column of Biogel P-150 (80 cm × 1.5 cm I.D.) calibrated with alcohol dehydrogenase, yeast (M_r 150 000), concanavalin A (M_r 100 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 29 000) and cytochrome *c* (M_r 12 400). A 1.5-ml volume was collected in each tube, with 0.15 *M* phosphate-buffered saline (pH 7.2) containing 10 m*M* calcium chloride, 10 m*M* magnesium chloride and 10 m*M* manganese chloride at a flow-rate of 10 ml/h.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) of the purified samples were performed at pH 8.3 in 7.5% gel [11]. The protein staining was done with Coomassie Brilliant Blue. The molecular mass of the samples treated with sodium dodecyl sulphate (SDS) in presence of 2-mercaptoethanol was determined [12] using MW-SDS-70 molecular mass kit as standard marker.

Protein estimation

The protein was determined by the method of Lowry et al. [13], using bovine serum albumin as standard.

Hemagglutination assay

The hemagglutination assay was performed by standard serial dilution with Crook's microtiter system [14], by using a 2% erythrocyte suspension in 0.15 *M* phosphate-buffered saline (pH 7.2) containing 10 m*M* calcium chloride, 10 m*M* magnesium chloride and 10 m*M* manganese chloride. The agglutination was assessed after a 60-min incubation at 37°C. The activity was expressed as the titre, the reciprocal of the greatest dilution at which agglutination could be detected. Inhibitory activities of the carbohydrates and sialic acid were determined in a similar system with serial dilutions.

RESULTS

Table I lists the purification of the lectins with a concomitant increase in specific activity. PAGE of the crude extract displayed a mixture of stained zones with a few sharp bands; GF-1, GF-2 and albumin proteins also contained several proteins distinct from each other. Crude extracts of rice bean did not agglutinate the erythrocytes of rabbit, mice, rat, sheep, hamster and guinea-pig. However, it agglutinated human blood group A erythrocytes at low concentration (25 μg), and it agglutinated human blood group B erythrocytes at higher protein concentration (125 μg). In both cases the reactions were weak. The crude extracts were separated into three fractions, GF-1, GF-2 and albumin proteins, by the salt solubility pH-dependent method [15]. The fraction GF-1, most remarkably, possessed all the lectin activity present in the crude extract; fraction GF-2 and albumin

TABLE I

PURIFICATION OF LECTINS FROM RICE BEAN SEEDS (15 g)

N.d. = not determined; Nil = the experiment has been done but no lectin activity is observed.

Fraction	Total protein (mg)	Specific activity (titer/mg of protein)		Total activity (titer)		Yield (%)	Purification (fold)
		Group A	Group B	Group A	Group B		
Crude extract	1915.0	2.3	Negligible	4404.5	Negligible	100	1
Salt solubility pH-dependent							
GF-1	260.0	15.2	Negligible	3952.0	Negligible	89.72	6.60
GF-2	407.0	Nil	Nil	–	–	–	–
Albumin proteins	963.0	Nil	Nil	–	–	–	–
Chromatofocusing of GF-1							
P1 (blood group B specific)	60.0	Nil	7.2	–	432.0	N.d.	N.d.
P2	50.0	Nil	Nil	–	–	–	–
P3	75.0	Nil	Nil	–	–	–	–
P4 (blood group A specific)	65.0	50.0	Nil	3250.0	–	82.23	21.74
HPLC of							
P1	12.1	Nil	29.8	–	360.58	N.d.	N.d.
P4	3.9	727.0	Nil	2835.3	–	64.4	316.08

proteins were devoid of any lectin activity. Further resolution of GF-1 by chromatofocusing gave four peaks: P1, P2, P3 and P4. Lectin activity was observed in the fractions under P1 and P4 (Fig. 1). The P4 fraction showed greater affinity for A erythrocytes than did the P1 fraction. However, the P1 fraction reacted strongly with B erythrocytes only. The peak P1 was resolved on HPLC assay into nine peaks (Fig. 2). Similarly the peaks P2, P3 and P4 were resolved into nine, eight and five peaks, respectively (Figs. 3–5). The peaks were collected manually and lyophilized. To check the proper collection of peaks, each peak was injected separately onto the HPLC column and recovered.

In Fig. 5 the protein under peak IV possessed a very strong human blood group A specific lectin-I activity. The protein purification increased from 21.74-fold to 316.08-fold in this step with a specific activity of 727.

The HPLC assay of the protein under peak P1 in Fig. 1 yielded several peaks (Fig. 2) and the protein under peak VI (Fig. 2) showed blood group B specific lectin-II activity. However, the specific activity of lectin-II was very much less than the specific activity of lectin-I. The molecular masses of lectin-I and lectin-

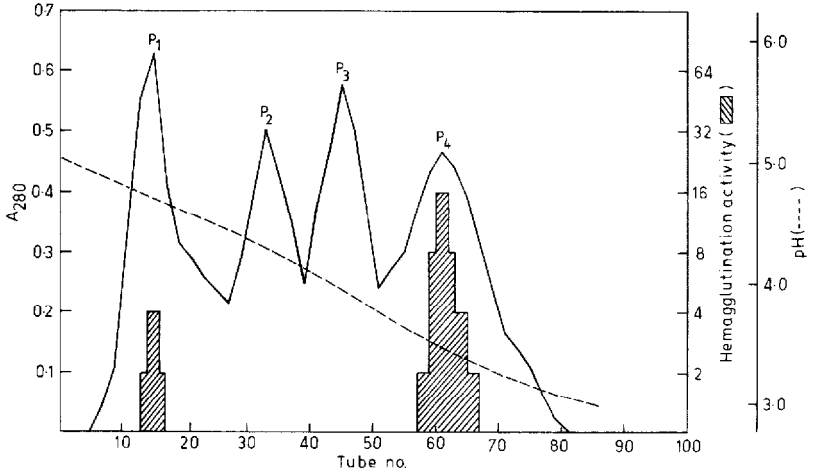

Fig. 1. Separation of the GF-1 fraction of rice bean by chromatofocusing as described in the text.

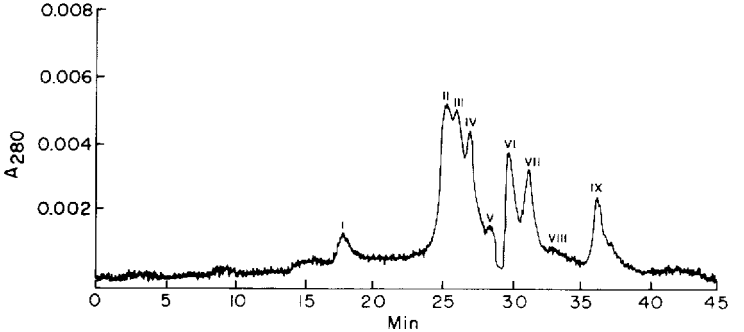

Fig. 2. HPLC assay of proteins from the peak P1 obtained from chromatofocusing. The sample protein (25 μg in 100 μl) was applied to the column. The column effluent was monitored at $A_{280}=0.02$ a.u.f.s./10 mV.

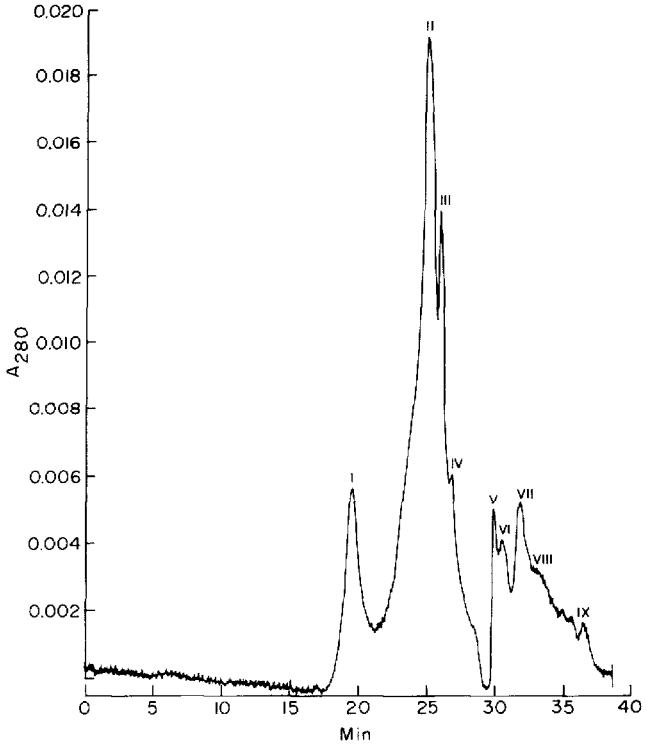

Fig. 3. HPLC assay of proteins from the peak P2 obtained from chromatofocusing. The sample protein (25 μg in 100 μl) was applied to the column. The column effluent was monitored at $A_{280}=0.02$ a.u.f.s./10 mV.

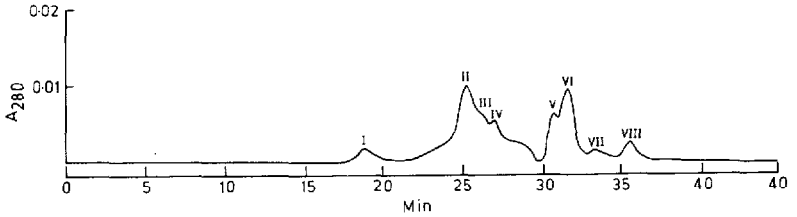

Fig. 4. HPLC assay of proteins from the peak P3 obtained from chromatofocusing. The sample protein (50 μg in 100 μl) was applied to the column. The column effluent was monitored at $A_{280}=0.1$ a.u.f.s./10 mV.

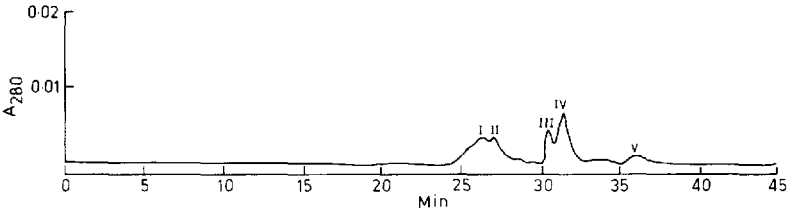

Fig. 5. HPLC assay of proteins from the peak P4 obtained from chromatofocusing. The sample protein (50 μg in 100 μl) was applied to the column. The column effluent was monitored at $A_{280}=0.1$ a.u.f.s./10 mV.

TABLE II

INHIBITION OF HEMAGGLUTINATIONS BY SUGARS

Sugars	Minimum concentration (m*M*) required to inhibit the hemagglutination activity	
	Blood group A specific lectin-I	Blood group B specific lectin-II
Glucose	3.45	4.2
N-Acetyl-D-glucosamine	1.95	2.92
Galactose	1.60	0.90
N-Acetyl D-galactosamine	0.45	1.73
Mannose	2.47	3.65
L-Arabinose	12.65	18.0
Fucose	120*	120*
Xylose	120*	120*
Sialic acid	120*	120*

*No inhibition below 120 m*M*.

II as determined by gel permeation on Biogel P-150 were 80 000 and 100 000, respectively. SDS-PAGE of the purified lectin-II demonstrated the monomer to have a molecular mass of 25 000. Lectin-I was dimeric in nature, with molecular masses of 18 000 and 22 000, respectively. The molecular masses were determined with respect to marker proteins used.

Table II indicates the effect of haptenic saccharide inhibition on erythroagglutination. GalNAc was the most potent inhibitor of the purified blood group A specific lectin-I and galactose was the specific inhibitor of the purified blood group B specific lectin-II.

It is interesting to note that protein under peak V in Fig. 4 contained an endogenous lectin inhibitor protein of molecular mass 40 000. This endogenous protein (15 μg) reacted very strongly (100% inhibition of agglutination) with blood group A specific lectin-I, whereas 10% inhibition of agglutination was caused by the same amount of protein with blood group B specific lectin-II. SDS-PAGE of the inhibitor protein demonstrated the monomer of this protein to have a molecular mass of 20 000.

DISCUSSION

In blood banks, lectins are used in blood typing, in detecting secretors and in testing erythrocyte polyagglutinable states [16,17]. Recently a human erythrocyte specific lectin from the seeds of Inidan coral tree, *Erythrina variegata* (Linn.). var. *orientalis* (Linn.) Merrill has been shown to be of importance in immunohematology [18,19]. *Saliva selarea* seed lectin has been characterized and shown to agglutinate specifically Tn erythrocytes, and also, at higher concentrations, Cad erythrocytes [20].

Two GalNAc/galactose binding lectins, agglutinating human blood group A and B erythrocytes, respectively, were purified from the seeds of *P. calcaratus*.

Lectin-I, with a molecular mass of 80 000, had greater affinity for GalNAc binding and showed specificity towards blood group A erythrocytes. It contained two promoters with molecular masses of 18 000 and 22 000. The other purified lectin, lectin-II, was blood group B specific, with a molecular mass of 100 000. It contained a single promoter with molecular mass of 25 000 and exhibited a greater affinity towards D-galactose. Distinct lectins have also been reported from the seeds of *Vicia cracca, Ulex europaeus* and *Griffonia simplicifolia* [1].

The endogenous lectin-binding protein of molecular mass 40 000 affected the lectin-I activity more than the lectin-II activity. This protein was possibly a dimer of the subunit, molecular mass 20 000. The endogenous lectin inhibitor proteins have been discussed in a recent review [21].

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