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Lectin from rice

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Abstract. N-Acetyl-D-glucosamine-binding lectin was isolated and purified from rice by ammonium sulphate fractionation and affinity chromatography using N-acetyl-D-glucosamine linked Sepharose 6B column. It gave a single hand on Polyacrylamide disc gel. It was identified as a glycoprotein. The purified lectin dissociated into two components on Sephadex G-100 column chromatography,–a higher molecular weight fraction not containing any carbohydrate and a lower molecular weight glycoprotein fraction. The apparent molecular weights of these fractions were 85,000 and 14,500. The lectin agglutinated erythrocytes of human A,B,O groups and of several other mammals and its activity was inhibited only by N-acetyl-D-glucosamine. The glycopeptide isolated by pronase digestion of the lectin was homogeneous and did not possess agglutinating activity. It contained about 10% carbohydrate of which xylose, arabinose and glucose were the major components.

Keywords. Rice lectin; affinity chromatography; glycoprotein; glycopeptide; reversible hemagglutination.

Introduction

Lectins are carbohydrate binding proteins. These agglutinate erythrocytes and other types of cells (Sharon and Lis, 1972). Lectins have been used in the studies of membrane structure, function and cell transformation (Lis and Sharon, 1973; Nicolson, 1974; Rapin and Burger, 1974). Purification and properties of lectins have been reviewed in detail by Goldstein and Hayes (1978). Since many lectins have affinity towards sugars, this property of the lectins has been utilised for their purification by affinity chromatography. A special account of N-acetyl-D-glucosamine-binding lectins has been dealt by Caderburg and Gray (1979). The present study deals with the isolation, purification by affinity chromatography and describes a few properties of an N-acetyl-D-glucosamine binding lectin from an Indian variety of rice.

Materials and methods

Materials

Rice, S-701 and other varieties, used in these experiments were obtained from Visweswaraiah Canal Farm, Mandya, Karnataka.

Epoxy-activated Sepharose 6B and Sephadex G-100 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, N-Acetyl-D-glucosamine, other sugars used, bovine serum albumin, ovalbumin, trypsin and lysozyme were purchased from Sigma Chemicals Co., St. Louis, Missouri, USA. Pronase was a Kaken Chemicals' product, Tokyo, Japan. AU the other chemicals used were of the analytical grade.

Human A,B,O group erythrocytes were obtained from the Blood Bank of the local hospital. Erythrocytes of other experimental animals were collected from the animal house, Central Food Technological Research Institute, Mysore.

Methods

Synthesis of N-acetyl-D-glucosamine Sepharose 6B: This biospecific adsorbant was prepared according to the procedure of Vretblad (1976) by coupling N-acetyl-D-glucosamine to epoxy-activated Sepharose 6B.

Preparation of the crude extracts of rice: Ten different varieties of rice were screened for the hemagglutinating activity by the following procedure. Cleaned paddy was dehusked and the rice obtained was powdered and passed through an 85-mesh seive. The flour was defatted by repeated extraction with cold petroleum ether and air-dried. Defatted rice flour was dispersed in 0.05 M sodium citrate buffer of pH 4.0 in the ratio of 1:5 (w/v), stirred in cold for 3 h and centrifuged at 2000 g for 20 min. The clear supernatant obtained was dialysed against phosphate-buffered-saline (PBS), pH 7.4 (0.006 M) and checked for the hemagglutinating activity.

Isolation of the crude rice lectin from S-701 variety: Crude lectin was precipitated by adding solid ammonium sulphate to 50% saturation to the supernatant prepared as described above from S-701 variety rice. The precipitate recovered by centrifugation was dissolved in a minimum amount of 0.05 M sodium acetate buffer, pH 4.5 and dialysed extensively against the same buffer.

Purification of the lectin on the biospecific adsorbant: Fifteen of N-acetyl-D-glucosamine-linked Sepharose 6B was packed in a column (16 × 60 mm) and was equilibrated with 0.05 M sodium acetate buffer, pH 4.5. Four ml of the crude lectin (2.5 mg/ml) was loaded and allowed to flow through the column at a rate of 10 ml/h which was maintained throughout the experiment. The unadsorbed protein was washed with 0.05 M acetate buffer (pH 4.5) till the effluent showed no absorbance at 280 nm. The bound lectin was then eluted from the column with N-acetyl-D-glucosamine (60 mg/ml). The emergence of the lectin was monitored by measuring absorbance at 280 nm. Appropriate fractions were pooled, concentrated by ammonium sulphate precipitation at 50% saturation, dissolved in a minimum amount of phosphate-buffered-saline and dialysed extensively against the same buffer.

Hemagglutination assay: Hemagglutination assay was carried out by the serial dilution technique with trypsinized rabbit-erythrocytes (Us and Sharon, 1972). The highest dilution of the lectin causing visible agglutination was identified as the titre value. The hemagglutinating activity of the lectin against trypsinized erythrocytes of different species was examined by adopting the same procedure.

Polyacrylamide disc gel electrophoresis: Electrophoresis was carried out in 7.5% gels using 0.005 M Tris-glycine buffer, pH 8.3, according to the method of Ornstein (1964). The gels were stained with 0.1% amido black in 7% acetic acid and destained in 7% acetic acid solution. The presence of the glycoprotein was detected by staining the gels with periodic acid-Schiff's reagent and destaining by using 1% sodium metabisulphite.

Molecular weight determination: The molecular weight of the purified rice lectin was determined from the data obtained by gel filtration on Sephadex G-100. Bovine serum albumin, ovalbumin, trypsin and lysozyme were used as the reference proteins. Sephadex G-100 column (1.5 × 81 cm) was equilibrated with phosphate-buffered saline. Four ml of the purified lectin (2 mg/ml) in the buffer was loaded on to the column and eluted with the same buffer. Fractions (2 ml) were collected at a flow rate of 15 ml/h. Protein absorbance was measured at 280 nm. Elution of the other proteins was similarly carried out. A calibration curve was obtained by plotting log molecular weights of the reference proteins vs their V_e/V_o values.

Sugar inhibition studies: Inhibition of hemagglutination by various sugars was tested as follows. The purified lectin (5 μg) in 0.1 ml of phosphate-buffered-saline was serially diluted with the same buffer. To each of the pits 0.1 ml of the test-sugar was added and incubated for 1 h at 37°C. Later 0.2 ml of 2% trypsinised-rabbit-erythrocytes were added, kept at 37°C for 1 h and observed for hemagglutination.

Reversibility of hemagglutination by the inhibitory sugar (N-acetyl-D-glucosamine) was performed according to the procedure of Lis *et al.* (1970).

Preparation of the glycopeptide: The lectin was digested exhaustively with pronase by the procedure of Kawai and Anno (1975). The undigested protein and the added enzyme were precipitated with trichloroacetic acid and removed by centrifugation. The glycopeptide was precipitated from the supernatant by adding four volumes of 95% alcohol in the presence of 1% potassium acetate (Kirk and Derbye, 1957). It was repeatedly washed with alcohol and dried *in vacuo.*

Sugar composition of the glycopeptide: The total carbohydrate content of the glycopeptide was estimated by phenol-sulphuric acid reagent (Dubois *et al.,* 1956).

Qualitative examination of sugars

The sugar composition of the glycopeptide was determined by hydrolysing 2 mg of it with 2 ml of 2 N sulphuric acid for 6 h in a boiling water-bath. It was neutralized with barium carbonate and fractionated on Dowex-50 (H^+) and Dowex-2 (formate) columns (Simkin *et al.,* 1964). The neutral sugar fraction was eluted with water and concentrated *in vacuo* at 40°C to remove HCl. The neutral and aminosugars were identified by paper using *n*-butanol: pyridine: water system (6 :4: 3 v/v). The chromatograms were developed for 36 h and sprayed separately with alkaline silver nitrate to identify the neutral sugars and alkaline acetyl acetone reagent for aminosugars.

Quantitative estimation of neutral sugars

Quantitative estimation of neutral sugars was carried out after their separation by chromatography on freshly prepared potato starch column (Gardell, 1953). The column (0.9 × 26 cm) was equilibrated with 200 ml of *n*-bulanol: *n*-propanol:water (4:1:1 v/v). The neutral sugar fraction obtained from 9 mg of glycopeptide was loaded on to the column and eluted with the above solvent mixture. Fractions (1 ml) were collected. One-half of each fraction was evaporated separately *in vacuo* and analysed for sugar by Nelson- Somogyi method (Nelson, 1944; Somogyi, 1952). The fractions containing individual sugars were pooled, evaporated to dryness and identified by paper chromatography to be xylose, arabinose and glucose. The elution pattern of the test sample was compared with that of the standard sugar mixture containing the sugars suspected to be present in the test sample.

Results and discussion

Hemagglutinating activity in the crude extracts of Indian varieties of rice

The presence of lectin in a Japanese variety of rice (*Akibare*) was first reported by Takahashi *et al.* (1975). These workers purified the lectin by ammonium sulphate fractionation and ion-exchange chromatography on DEAE- and CM-celluloses. Its molecular weight, amino acid composition, sugar composition etc., were studied. However, they have not reported on the sugar inhibition of hemagglutination by the lectin.

Extracts of several Indian varieties of rice were examined for the presence of hemagglutinating activity, of which S-701 variety contained the maximum activity (table 1). The lectin from this variety of rice was partially purified by adding ammonium sulphate to the extract to 50% saturation. The activity of this crude lectin was specifically inhibited only by N-acetyl-D-glucosamine. This affinity of the lectin for this sugar was utilised for its further purification.

Table 1. Hemagglutinating activity of the crude extracts of different Indian varieties of rice.

Variety tested	Titre value/ 0.2 ml of the crude extract
IET-2724	1
IET-2730	1
S-701	4

Hemagglutinating activity was not present in C-435, CH-45, IET-3232, Mahsuri, MR-301, Pushpa, SR-26B and Sona varieties of rice.

Purification of lectin

When the crude lectin was passed through the N-acetyl-D-glucosamine-Sepharose 6B column, the lectin was bound to the biospecific adsorbant. This was shown by the absence of activity in the unadsorbed portion of the protein removed by washing with 0.05 M acetate buffer, pH 4.5. The bound-lectin could be eluted by sodium acetate buffer containing N-acetyl-D-glucosamine yielding a pure protein (peak 2, figure 1).

The lectin purified by the affinity chromatography showed only one band (figure 2) on Polyacrylamide gel electrophoresis and staining with amido black, whereas the crude extract gave 6 bands (figure 2). The pure lectin was also stained with periodic acid- Schiffs reagent showing that it was a glycoprotein.

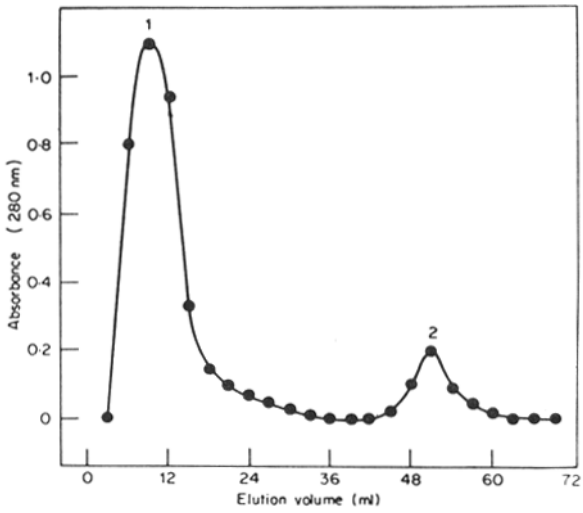
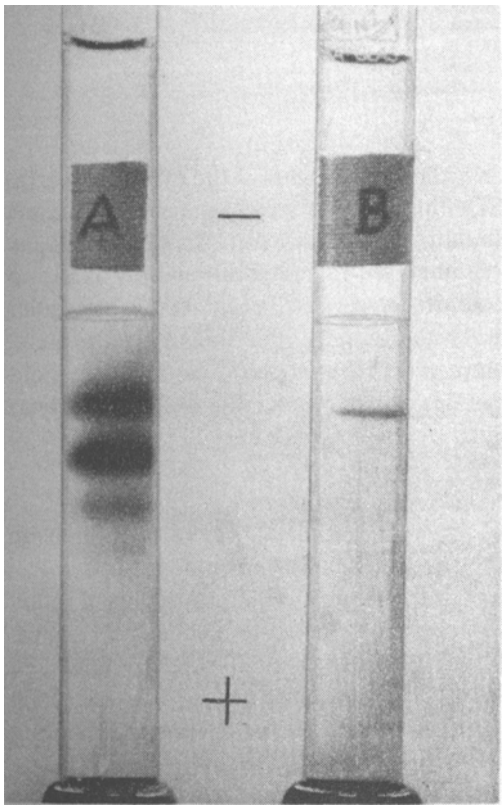


Figure 1. Afinity chromatography of the crude rice lectin. 4 ml of the crude lectin (2.5 mg/ml) was applied to N-acetyl-D-glucosamine linked Sepharose 6B column and was washed with 0.5 M acetate buffer of pH 4.5 at a flow rate of 10 ml/h till the eluant showed no absorbance at 280 nm. The column was eluted with N-acetyl-D-glucosamine (60 mg/ml) in the same buffer. Peak 1 denotes the unadsorbed protein and peak 2 the purified rice lectin.



Figured 2. Polyacaryaminde gel electrophorectic pattern of rice lectin. A crude rice lectin (50% ammonium sulphate fraction). B lectin purified by affinity chromatography no N-acetyl-D-glucosamine-linked sepharose 6B cooumn.

Dissociation and molecular weight of the lectin

The purified lectin eluted as two peaks from Sephadex G-100 column using phosphate-buffered-saline as the eluant and the activity was found in both the fractions figure 3) This anomalous behaviour was also observed in the case of ulex and lotus lectins

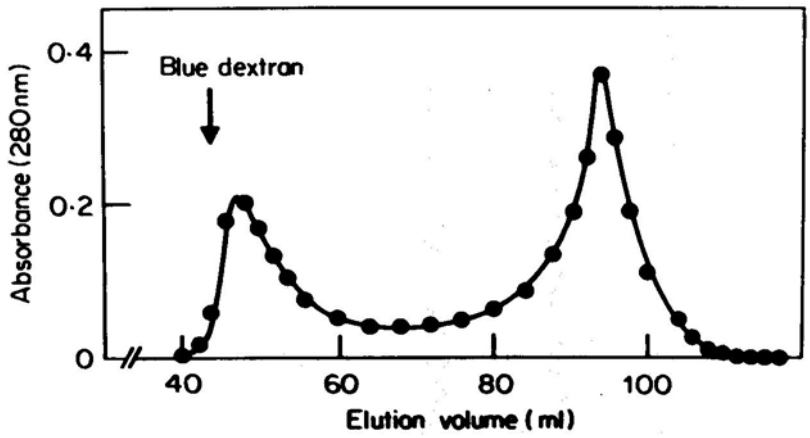


Figure 3. Sephadex G-100 column chromatography of the purified rice lectin. 8 mg of the pure lectin in 4 ml of phosphate-buffered-saline was applied to a Sephadex G-100 column (1.5 × 81 cm). The column was eluted with phosphate-buffered-saline at a flow rate of 15 ml/h. 2 ml fractions were collected and absorbance mesured at 280 nm. Peak 1 noncarbohydrate and peak 2 carbohydrate containing fractions.

(Allen and Johnson, 1977). The apparent molecular weights of the two fractions (as determined on G-100 column calibrated with molecular weight markers-lysozyme, trypsin, ovalbumin and bovine serum albumin) were 85,000 and 14,500 respectively. The lower molecular weight protein contained the carbohydrate moiety as shown by the phenol-sulphuric acid test. Takahashi *et al.* (1973) reported a molecular weight of 10,000 for the rice lectin from *Akibare* variety which was a glycoprotein. In this respect, the lectin from S-70 is interesting in that, a part from the lower molecular weight glycoprotein fraction, it contains a higher molecular weight non-glycoprotein fraction.

Effect of carbohydrate on the hemagglutinating action

The lectin agglutinated trypsinized and untrypsinized erythrocytes of rabbit, rat, brown mice, hamster, guinea pig, monkey and human A,B,O blood groups.

The agglutinating activity of the lectin against trypsinized erythrocytes of rabbit was not inhibited by D-xylose, D-arabinose, D-fucose, L-fucose, D-glucose, D-galactose, D-mannose, D-glucosamine, D-galactosamine, N-acetyl-D-galactosamine, N-acetyl neuraminic acid, maltose, lactose, sucrose, melibiose and melizitose even at 100 mM concentration. Only N-acetyl-D-glucosamine could inhibit the agglutination at 1 mM concentration (table 2).

The agglutinated-rabbit-erythrocyte clot could be reversed completely only by N-acetyl-D-glucosamine at 300 mM concentration (data not shown).

Table 2. Inhibition of haemagglutination by sugars.

Test sugar	Sugar concentration (mM)	Titre
Lectin alone	—	64
D-glucosamine	100	64
D-galactosamine	100	64
N-acetyl neuraminic acid	100	64
N-acetyl-D-galactosamine	100	64
N-acetyl-D-glucosamine	1	16
	10	8
	100	0

Preparation and sugar composition of the glycopeptide

The glycopeptide prepared by pronase -digestion of the lectin was homogeneous on Polyacrylamide gel electrophoresis. It had no agglutinating activity against rabbit erythrocytes. On the other hand, the lectin digested by chymotrypsin for 24 h did not show significant loss of activity. These experiments suggest that extensive digestion of the lectin resulted in the loss of activity whereas limited breakage of the peptide bonds does not affect the activity.

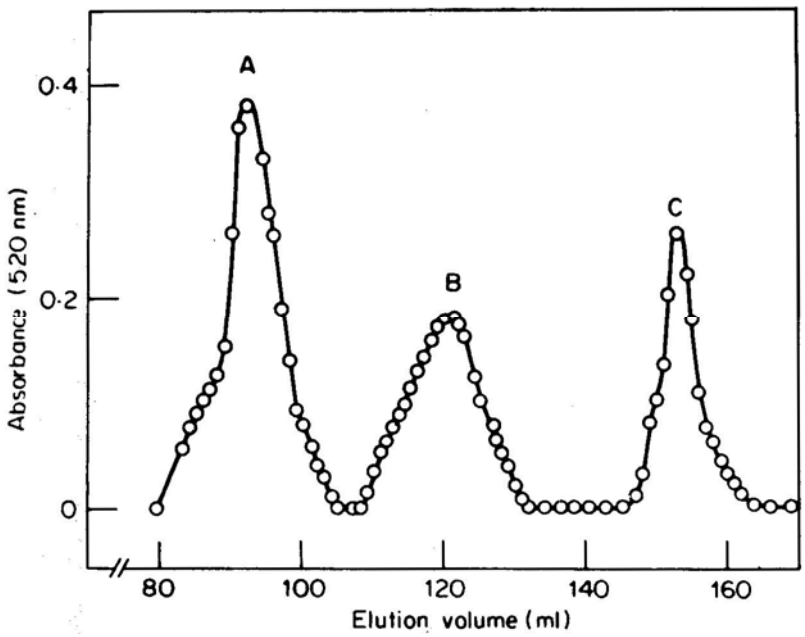


Figure 4. Column chromatography of neutral sugars of the glycopeptide. Neutral sugar fraction obtained from 9 mg of the glycopeptide was applied to a starch column and eluted with *n*-butanol:*n*-propanol:water mixture (4:1:1 v/v) at a flow rate of 2 ml/h. 1 ml fractions were collected. Peaks A, B and C represent xylose, arabinose and glucose respectively.

The glycopeptide contained about 10% cabohydrate. Xylose, arabinose and glucose constitute the neutral sugar fraction as shown by paper chromatography. No aminosugars were detected by paper chromatography. The three neutral sugars could be separated and estimated quantitatively on a starch column (figure 4).

Table 3. Carbohydrate composition of the glycopeptide prepared from rice lectin.

Sugar	mol/100 mg of the glycopeptide
Xylose	27.8
Arabinose	22.2
Glucose	11.1

Xylose, arabinose and glucose were present in the molar ratio of 2.5:2.1 (table 3). Takahashi *et al.* (1973) reported that the rice lectin they prepared contained glucose, mannose, xylose and glucosamine in the ration of 13:1:1:1. In this respect also, the lectin from variety S-701 was different in that it lacked mannose and glucosamine but contained arabinose. Thus the lectin from S-701 seemed to be primarily a pentose containing glycoprotein.

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