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Reinvestigation on the Purification and Characterization of Rice-bran Lectin

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Abstract: Two lectins from the rice bran of Amon variety were purified by successive chromatography of 100% ammonium sulfate saturated crude extract on Sephadex G-50 followed by ion-exchange chromatography on DEAE-cellulose. Both the lectins are found to be homogenous as judged by polyacrylamide gel electrophoresis. The molecular weight of the lectins, RBL-1 and RBL-2 as determined by gel filtration and SDS-PAGE were estimated to be as 22.000 and 36.000 respectively. The lectins are dimer and their subunits are held together by disulfide bonds. The lectins agglutinated rat red blood cells and the agglutination was inhibited specifically by D-glucose, D (+) glucosamine-HCl and N-acetyl-D-glucosamine. The lectins are glycoprotein in nature with neutral sugar content of 4-5.4%. The lectins RBL-2 are more cytotoxic than RBL-1 as observed by brine shrimp nauplii mortality rate bioassay.

Key words: Lectin

Introduction

Rice bran is the most important by product of rice milling industry and obtained to the extent of 3-10% of milled rice, depending on the degree of polishing. The protein content in the true rice bran ranges from 9.8-15.4% and that in defated bran from 10.4 to 21%. Lectins are glycoproteins, which are valuable as specific molecular probes for carbohydrate in studies of glycosylated materials and the cell membranes. Although hundreds of lectins are known, the structures of most lectins have not yet been fully elucidated and supplies of their sources are often limited. It was reported that rice-germ is a rich source of the lectin (Sun and Tang, 1981). Lectins from rice-bran were purified so-far at least from four different laboratory but the result are quite contradictory although hemagglutination of the lectin was inhibited specifically by N-acetyl glucosamine (Takahashi *et al.*, 1973; Tsuda, 1979; Shen *et al.*, 1984).

The present paper describes the reinvestigation on the purification and characterization of lectin from rice-bran of Amon variety, a widely cultivated variety in Bangladesh, and also compared the present data to some extent with the reported data of other laboratory.

Materials and Methods

Rice bran of Amon variety from cottage type huller mills was collected from Noor-Habib rice mills, Bangladesh. After collection of bran, it was cleaned and threshed manually by winnowing fan to remove seed coat or husk. Then the germ was separated from broken grain by sieving. The cleared germ was dried in the sun and used for experimental purposes. Sephadex G-50 and DEAE-cellulose were the products of Sigma Chemical Co. USA. The molecular weight markers were purchased from Fluke Chemical, Switzerland. All the other reagents used were of analytical grade.

Purification of Rica bran lectin: Preparation of fat-free meal:- The rice-germ was pounded uniformly into fine powder with mortar and pestle. This powder was mixed with pre-cooled petroleum ether and homogenized uniformly by a homogenizer at 4°C. The oily homogenate mixture was then stirred slowly at 4°C for overnight. The homogenate was filtered through a double layer of clean muslin cloth. The process was repeated at least for 2-times in order to make homogenate quite free of lipids. Finally the filtrate was clarified further by centrifugation at 10,000 g, 4°C for 15 minutes. The ppt obtained after centrifugation was collected and dried at 40°C to remove the

petroleum ether.

Preparation of Crude Protein Extract: The fat-free dry-powder was mixed uniformly with pre-cooled distilled water containing 0.15 M NaCl, pH 5.4 (pH was adjusted by 0.1 M HCl 6 ml/gm meal) in a beaker and kept overnight at 4°C with occasional stirring. The suspension was then filtered through double layer of muslin cloth in the cold room. The filtrate was collected and clarified further by centrifugation at 10,000 g, 4°C for 20 minutes. The clear supernatant was then adjusted to 100% saturation by solid ammonium sulfate. The ppt was collected by centrifugation at 10,000 g, 4°C for 10 minutes. The precipitate obtained was dissolved in minimum volume of pre-cooled distilled water and dialyzed against 5 mM phosphate buffer pH 7.6 for 24 hours at 4°C. After removal of insoluble material by centrifugation, the clear supernatant was used as crude protein extract.

Gel filtration: Gel filtration of crude protein extract was performed on Sephadex G-50 using 5 mM phosphate buffer pH 7.6 at 4°C.

DEAE- cellulose column chromatography: The active protein fraction (F-1), obtained after gel filtration, was dialyzed against 10 mM Tris-HCl buffer, pH 8.4 for overnight and then loaded onto the column at 4°C. The protein from the column was eluted by stepwise increases of NaCl in the same buffer.

Polyacrylamide gel electrophoresis: Polyacrylamida disc electrophoresis was conducted at room temperature, pH 8.4 on 7.5% gel as described by Ornstein (1964) and 1% amido black was used as staining reagent. For sugar detection, gels were stained according to the procedure of periodic-acid Schiff = (PAS) reagent as described by Zacharius *et al.* (1969).

Molecular weight determination

a) Gal filtration: The molecular weight of the proteins was determined by Gel filtration on Sephadex G-50 following the procedure as described by Andrews (1965). Lysozyme (Mr 14.6 KID), trypsin inhibitor (Mr 20 KO), egg albumin (Mr 45 KD) and bovine serum albumin (Mr 67 KU) were used as reference proteins.

b) SDS-PAGE: SDS polyacrylamide gel electrophoresis was conducted at room temperature, pH 7.2, according to the method of Weber and Osborn (1966). The marker proteins used were same as those used for gel filtration Dissociation

and reduction of proteins were performed by heating for 3 minutes at 100°C in 0.1% SDS with 0.5% β -mercaptoethanol and the proteins are stained with bromophenol blue.

Hemagglutination & Hemagglutination inhibition studies: Hemagglutinating activity was assayed by using 2% albino rat red blood cells as described by Lin *et al.* (1981). The protein solution (0.2 ml) in 5 mM phosphate buffer saline, pH 7.2 was mixed with 0.2 ml of 2% rat blood cell and incubated at 37°C for an hour. The hemagglutinating inhibition test was also performed in the presence of different saccharides following the procedure as described above.

Protein end Carbohydrate analysis: The concentration of protein was assayed by the method of Lowry *et al.* (1951) using BSA as standard. The neutral sugar contents of the lectins were estimated by phenol-sulfuric acid method as described by DuBois *et al.* (1956) with D-glucose as the standard.

Cytotoxicity: Cytotoxicity was studied using Brine shrimp following the procedure as described by Meyer *et al.* (1982).

The procedure is as follows:

A. Preparation of sea salt: 38 gm sea salt was weighed, dissolved in one liter of distilled water and then filtered off.

B. Hatching of brine shrimp eggs: Sea water was taken in a small divided glass tank and shrimp eggs were added to one side of the divided tank and this side was covered. The shrimps were allowed for two days to hatch and mature as nauplii (larvae). The hatched shrimps were attracted to the lamp on the other side of the divided tank through the perforations in the dam. These nauplii were taken for bioassay.

C. Preparation of sample solution: 2 ml of each proteins in TrisHCl buffer pH 8.4 (conc. 1 mg/ml) were dialyzed separately against distilled water for 3 hours at 4°C.

D. Application of test solution and nauplii in the vials: At room temperature 10, 25, 50, 100, 150 and 200 μ l of the stock solution were taken in vials and 5 ml of the sea water was added to each vial containing 10 brine shrimp nauplii. So, the concentrations of the sample in the vials were 2, 5, 10, 20, 30 and 40 μ g/ml respectively. Three vials were used for each concentration and control was used containing 10 nauplii in 5 ml of seawater.

Counting of nauplii: After 24 hours the vials were observed and the number of survivors in each vial was counted and noted. From this data, the percentage of mortality of the nauplii was calculated at each concentration.

Results

Purification of lectin: The crude protein extract from rice bran was applied to Sephadex O-50 column at 4°C previously equilibrated with 5 mM phosphate buffer, pH 7.6. As shown in Fig. 1, the component of the crude extract was eluted as one major peak i.e. F-1 and one minor peak i.e. F-2. It was found that only the F-1 fraction possessed the major hemagglutinating activity. The fraction, F-1 as indicated by solid bar was pooled, precipitated to 100% saturation by ammonium sulfate and purified further by ion-exchange chromatography on DEAE-cellulose. The fraction, F-2 was not used for further study as it contained mostly colored materials and small amount of very low molecular weight protein.

Ion-exchange chromatography: The ammonium sulfate ppt. of

F-1 fraction was dissolved in minimum volume of distilled water and dialyzed against 10 mM Tris-HCl buffer, pH 8.4 at 4°C for 24 hours. After centrifugation, the clear supernatant was applied to DEAE-cellulose column, which was previously equilibrated with the same buffer, and the protein was eluted from the column with the buffer containing different concentration of NaCl. As shown in Fig. 2 the component of F-1 were separated into two minor peaks, F-1a and F-1b and two sharp peak's, F-1c and F-1d, which were eluted by the buffer containing 0.03 M, 0.06 M, 0.12 M and 0.25 M NaCl respectively.

It was found that the fraction F-1c and F-1d contained the bulk of the hemagglutinating activity while F-1a and F-1b fraction contained very little activity, which may be due to contamination of the proteins of latter peaks, or some other proteins. Only the very active fractions F-1c and F-1d were used for further study and their homogeneity were determined by polyacrylamide disc gel electrophoresis. Both the fractions might be contained pure protein as they gave single band on polyacrylamide gel (Fig. 3).

A brief summary of the overall purification steps of rice-bran lectins is presented in Table 1. It was found that the specific activities of the different protein fractions were increased after each purification steps. Although the overall yield of the activity of pure proteins (F-1c and F-1d) were 41.2% and over 96% of the extracted protein was destroyed during purification steps but the purification fold of the proteins were increased to about 25 fold. The decrease in yield may be due to denaturation of proteins during the lengthy purification procedure or some other reasons.

Molecular weight determination: The molecular weight of F-1c (Rice bran lectin-1, RBL-1) and F-1d (Rice bran lectin-2, RBL-2), as determined by gel filtration on Sephadex O-50 were estimated to be 22,000 and 36,000 respectively. Under non-reducing condition the molecular weight of the RBL-1 and RBL-2 as determined by SDS-PAGE were estimated to be 21,500 and 35,000 respectively. In presence of 0.1% SDS and 0.5% 13-mercaptoethanol, RBL-1 produced two separate bands with molecular weight of 11,500 and 10,000 and RBL-2 also produced two bands with Molecular Weight of 20,500 and 14,500 (Fig. 4).

Hemagglutinating activity: Both the lectins agglutinated specifically rat red blood cells and the minimum concentration of RBL-1 and RBL-2 needed for visible agglutination were 8.5 μ g ml⁻¹ and 10.1 μ g ml⁻¹ respectively. The agglutination of RBL-1 was inhibited by D-glucose and D (+) glucosamine-HCl while that of RBL-2 was inhibited by D-glucose and D (+) glucosamine-HCl and N-acetyl-D-glucosamine (Table 2).

Protein and Carbohydrate analysis: The lectin, RBL-1 and RBL2 produced pinkish red band on poly acrylamide gel when the gels are stained with periodic-acid Schiff = s staining reagent and gave yellow orange colour in the presence of phenol-sulfuric acid which indicating the presence of sugar and the neutral sugar content of the lectins were found to 4.0% and 5.4% respectively. The lectins, RBL-1 and RBL-2 in aqueous solution gave absorption maximum around 278 nm and 276 nm, and minimum around 248 nm and 242 nm and the absorbance of 1.0 at 280 nm were found to be equal to 0.85 mg and 0.95 mg of proteins by the Lowry method respectively.

Cytotoxicity: In the brine shrimp lethality bioassay, both the lectins showed positive results indicating that the lectins are cytotoxic in nature. The mortality rate or brine shrimp nauplii was found to be increased with the increase of concentration

Table 1: Purification of rice bran lectins

Fraction	Total Protein (mg)	Total Hemagglutination activity (titre)	Specific activity (titre/mg)	Yield (%)	Purification (fold)
Crude extract	650	1820	2.8	100.00	1.00
100% (NH ₄) ₂ SO ₄ Saturated	212	1208	5.7	66.37	2.03
Alter gel filtration	70	552	12.6	48.46	4.5
DEAE- Cellulose fractions					
F-1c	8	255	31.9	14.00	11.39
F-1d	13	495	38.1	27.20	13.61

Table 2: Hemagglutination-inhibition assay of Rice bran lectins by different sugars

Saccharides	Proteins			
	RBL-1		RBL-2	
	Conc. (mM)	Inhibition	Conc. (mM)	Inhibition
D-glucose	20		15	
D-mannose	110	-	110	-
D galactose	100	-	100	-
N-acetyl-	110	-	20	
D-glucosamine				
D-glucosamine-HCl	25		20	
Methyl (α)-	100	-	100	-
D-glucopyranoside				
Methyl (β)-	100	-	100	-
D-glucopyranoside				
N-acetyl-	110	-	110	-
galactosamine				

| = Inhibition

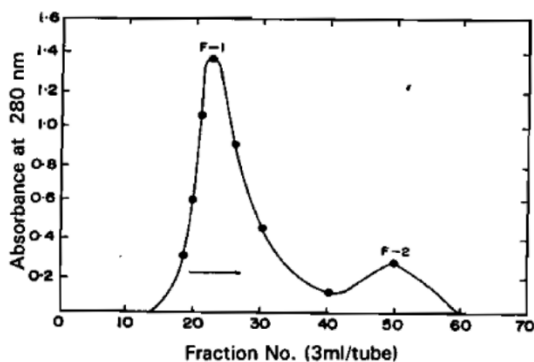


Fig. 1: Gel filtration of 100% (NH₄)₂SO₄ saturated crude protein extract on Sephadex G-50. The crude extract (62 mg) was applied to the column (2.5 × 100 cm) pre-equilibrated with 5 mM phosphate buffer, pH 7.6, at 4°C and eluted with the same buffer

of the sample and a plot of log of concentration vs. percent of mortality gave an almost linear correlation. From the graph, the C₅₀ (concentration at which 50% mortality of the brine shrimp nauplii occurred) was determined by extrapolation and the LC₅₀ values of RBL-1 and RBL-2 are 10 and 6.8 µg/ml respectively, The lectins, RBL-1 and RBL-2 were cytotoxic in nature since the mortality rate of brine shrimp naupli was increased with the increase in concentration of the lectins (Fig. 6).

Discussion

Although only one lectin was purified from some variety of rice bran by different laboratory but we purified first time two different lectins from rice bran of Amon variety. Both the lectins are glycoprotein in nature as they gave yellow orange colour in the presence of phenol-sulfuric acid. The presence of sugar in the lectins were further confirmed from the finding that they produced pinkish red band on polyacrylamide gel

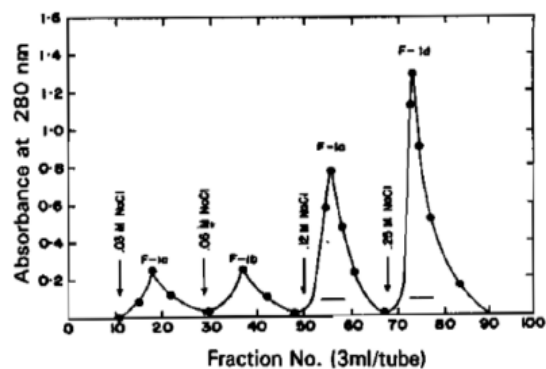


Fig. 2: Ion exchange chromatography of F-1 fraction on DEAEcellulose. F-1 fraction (32 mg) obtained by gel filtration, was applied to the column (2.1 × 24 cm) pre-washed with 10 mM Tris-HCl buffer, pH. 8.4 at 4°C and eluted by stepwise increases of NaCl concentrations in the same buffer



Fig. 3: Polyacrylamide disc gel electrophoretic pattern of the purified proteins at room temperature on 7.5% gel. (A = RBL-1 and B = RBL-2)

when the gels are stained with periodic-acid schiff's staining reagent after electrophoresis (Fig. 5). From the measurement of percentage of neutral sugar it seems that the rice bran lectins purified by our laboratory has been quite different from that purified in other laboratory. The rice bran lectin purified by Shen *et al.* (1984) contained only 0.8% carbohydrate while Takahashi *et al.* (1973) reported that rice seed hemagglutinin contained abnormally high amount of carbohydrate (26.8%). On the other hand Tsuda (1979) detected neither neutral nor amino sugars in rice bran lectin. Our research clearly indicated that both the rice bran lectins are glycoprotein with neutral sugar content of 4.0-5.4%.

From molecular weight and subunit measurements, the lectins purified in our laboratory are also found to be quite different from those of other laboratory. Although the values do not agree well we found the molecular weight of RBL-1 and RBL-2 to be about 22,000 and 36,000 by gel filtration and SDS-PAGE. Both the lectins are dimer in nature and their

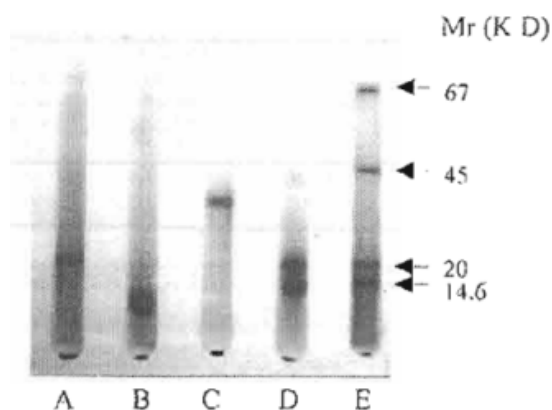


Fig. 4: SDS-polyacrylamide gel electrophoresis pattern of subunits of RBL-1 and RBL-2 at room temperature on 10% gel in presence and absence of 0.5% β -mercaptoethanol
 A = RBL-1, B = RBL-1 + 8-mercaptoethanol,
 C = RBL-2, D = RBL-2 + 13-mercaptoethanol,
 E = Marker proteins

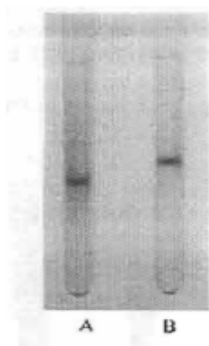


Fig. 5: Polyacrylamide disc gel electrophoretic pattern of the purified proteins (gels were stained with periodic-acid schiff's staining reagent) at room temperature on 7.5% gel, (A = RBL-1 and B = RBL-2)

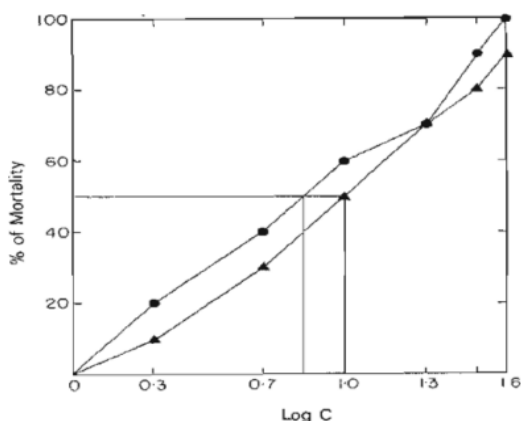


Fig. 6: Brine shrimp lethality bioassay. RBL-1 (\blacktriangle) and RBL-2 (\bullet)

subunits are held together by disulfide bonds. Shen *et al.* (1984) reported that under reducing and non-reducing condition the rice bran lectin gave three bands on the gel with molecular weight of 11,300, 13,700 and 19,000 while Tsuda (1979) estimated the molecular weight of rice bran lectin to be 44,000 by SDS-PAGE but by gel filtration on Sepharose 6B in 6M guanidine-HCl it was estimated to be 19,000 which was dissociated further into non-identical subunits (MW 11,000 and 8,200) if the lectin was reduced with gel filtration. On the other hand Takahashi *et al.* (1973) purified a rice seed hemagglutinin of MW 10,000.

The agglutinating activity of rice bran lectin, so far purified from different laboratory was inhibited by N-acetyl glucosamine only. In the present study, the lectins RBL 1 was found to be inhibited by D-glucose and D (+) glucosamine-HCl while that of RBL-2 was found to be inhibited by D-glucose, (+) glucosamine-HCl and N-acetyl-D-glucosamine.

Both the lectins, purified in the study are cytotoxic in nature as they affect significantly the mortality rate of brine shrimp. Rice bran lectin was also reported to be rnitogenic against mouse splenic and human peripheral lymphocytes (Tsuda, 1979; Takahashi *et al.*, 1973).

In conclusion, the rice bran lectins purified from Amon variety in the study are quite different from the rice bran lectin, purified so far by other laboratory. This may be due to use of different variety of rice bran as well as differences in techniques used for the purification of lectin.

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