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PURIFICATION OF THE RICE EMBRYO LECTIN AND ITS BINDING TO NITROGEN-FIXING BACTERIA FROM THE RHIZOSPHERE OF RICE

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A lectin was purified from rice embryos by aqueous acid extraction of crude embryo powder, followed by ammonium sulfate precipitation, affinity chromatography on agarose p-aminophenyl-β-D-N-acetylglucosamine and gel-filtration on AcA 54. Its homogeneity was checked by polyacrylamide gel electrophoreris, gel-filtration and immunological methods. The hemagglutinating activity of the purified rice lectin was 0.02 μg/ml. This lectin labelled with [^{14}C] acetic anhydride was shown to interact in vitro with different bacteria isolated from the rhizosphere of rice. The most efficient binding was obtained with *Beijerinckia V.*. The affinity constant K_a was (1.04 ± 0.30) x 10^7 M^{-1} and each bacterium contained 1660 ± 150 lectin receptor sites. In contrast, no interaction between bacteria isolated from the rhizosphere of maize or *E. coli K 12* and rice lectin was evidenced.

Lectins are carbohydrate-binding proteins widely distributed among plant and animal species (1-3). They can interact specifically with carbohydrate moieties of various glycoconjugates. It is interesting to note that the binding of plant lectins to plasma membrane glycoproteins in animal cells triggers many biological effects in these cells (4).

Although little is known about the endogeneous functions of plant lectins (5-7), the binding process between plant lectins and nitrogen-fixing bacteria has been widely studied (8). It was shown that a lectin from a particular legume only binds the corresponding rhizobial species and not bacteria infecting other such legumes. Thus, soybean lectin specifically binds the nodulating strains of *Rhizobium japonicum*, but not the non-nodulating strains (9, 10). This specific interaction has not been studied in gramineae, although they are essential in the world diet, and except for wheat germ agglutinin (3-6, 11), gramineae lectins have been poorly investigated. Studies of rice lectin are few and contradictory (12-14). This work describes the purification of a rice lectin and its specific interaction with nitrogen-fixing bacteria of the rhizosphere of rice.

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MATERIALS AND METHODS

The following chemicals were obtained from commercial sources : agarose p-aminophenyl-β-D-N-acetylglucosamine from the United States Biochemical Corporation ; AcA 54 from IBF (France) ; acrylamide, bisacrylamide and Coomassie brilliant blue R 250 from Biorad (U.S.A.) ; trypsin, N-acetylglucosamine and chitin from Sigma (U.S.A.) and [^{14}C] acetic anhydride (specific activity : 24 mCi/mmol) from Amersham (England). All other chemicals were of reagent grade (Merck, West Germany).

Rice (*Oryza sativa*) : the variety Balilla 28 was used and the rice embryo fraction was prepared in the Laboratory of Cereal Technology, I.N.R.A., Montpellier (France) by milling the seeds.

The **bacterial strains** used were : *Beijerinckia V.* (15) and *Azospirillum lipoferum 4 B* (16) isolated from the rhizosphere of rice, *Beijerinckia 39 J* and *Azospirillum lipoferum B7C* isolated from the rhizosphere of maize (17) in the Laboratory of Microbial Ecology of the Rhizosphere, Pedology Center, Nancy (France). *Escherichia coli K 12* was used as a control.

Affinity chromatography : 25 ml of agarose-p-aminophenyl-β-D-N-acetylglucosamine gel was washed with distilled water and equilibrated in 0.01 M Tris-HCl buffer containing 0.2 M NaCl, pH 8.0 and poured into a 2.2 x 8.4 cm column. The crude lectin fraction was layered on the column and after extensive washing with the equilibration buffer, elution was performed with 0.1 M sodium acetate buffer, pH 4.0, containing 0.2 M N-acetylglucosamine. Absorbance was read at 280 nm on each collected fraction (1.5 ml) and erythroagglutinating activity was determined.

Gel-filtration : the active fraction eluted from the affinity chromatography column was applied to an AcA 54 column (1.4 x 53 cm) in 0.1M sodium acetate buffer, pH 4.0, containing 0.2 M NaCl. Elution was carried out at 7 ml/h and 0.9 ml fractions were collected.

Electrophoresis : disc gel electrophoresis was carried out according to Reisfeld *et al.* (18) using 15 % polyacrylamide gel at pH 4.3 in acetic acid-β-alanine buffer, at 20°C under 2 mA/gel for 6h. The proteins were stained with Coomassie blue in 10 % acetic acid.

Erythrocytes : they were prepared from rabbit blood by repeated washings with phosphate buffered saline (PBS), pH 6.5. Trypsin-treated erythrocytes were prepared by incubating a 4 % cell suspension with 0.01 % trypsin for 1 h at 37°C followed by extensive washing with PBS.

Agglutinating assay : this was carried out in Cooke microplates. Serial dilutions of lectin in 50 µl of 0.15 M NaCl were mixed with 50 µl of a 4 % suspension of erythrocytes. After shaking and incubating for 30 min at 20°C, the agglutinating titer was determined visually.

Radiolabelling of rice lectin : the lectin was labelled with [^{14}C] acetic anhydride according to Miller and Great (19). The specific radioactivity of the labelled lectin was 6800 dpm/µg.

Interaction between rice lectin and bacterial cells : after the bacteria had been washed with 0.15 M KCl, 150 µl of a suspension containing 1×10^8 bacteria/ml was mixed with 100 µl of radiolabelled lectin (20 µg/ml) in PBS, pH 6.5, in the presence or absence of 0.15 M N-acetylglucosamine. After a 15 min incubation at 20°C, the bacteria were harvested by filtration on Whatman GF/C discs, with a Titretek multiple cell harvester. The discs were allowed to dry, put into glass vials and their radioactivity content was determined in an intertechnique spectrometer (France) using ACS (Aqueous Counting Scintillant, Amersham, England) as scintillation cocktail. The bound radioactivity was corrected for non-specific binding by subtracting the radioactivity bound to the bacteria in the presence of N-acetylglucosamine. Six separate measurements were performed for each of the five bacteria strains tested.

To determine the affinity constant and number of binding sites characterizing the interaction between rice lectin and the *Beijerinckia V.*

strain. Increasing concentrations of labelled lectin ranging from 3 to 100 μg/ml were added to a constant number of bacteria (1×10^8 cells/ml). Results were plotted according to Scatchard (20).

RESULTS

Purification of rice lectin.

Preliminary experiments had shown that more than 90 % of the agglutinating activity of rice seeds was located in the embryos, which were therefore used as a starting material.

Lipids of the rice embryo fraction (100g) were extracted at 20°C using chloroform-methanol 3/1 (v/v) as solvent.

The defatted rice powder was dried under a nitrogen stream, suspended in 0.5 l of 0.05 M HCl, 0.15 M NaCl, pH 1.3 and stirred overnight at 4°C. The insoluble material was pelleted by centrifugation and submitted to a second extraction under the same conditions. The supernatants were fractionated by ammonium sulfate at 4°C. More than 90 % of the hemagglutinating activity was precipitated at 50 % saturation. This "crude lectin fraction" was dialyzed against 0.01 M Tris-HCl buffer, containing 0.2 M NaCl, pH 8.0, and was submitted to affinity chromatography on an agarose-p-aminophenyl-β-D-N-acetylglucosamine column, since N-acetylglucosamine was shown to be a potent inhibitor of hemagglutination by rice lectin (14). Most of the protein material was eluted with the equilibration buffer (Fig. 1a). The hemagglutinating activity was recovered in a sharp peak eluted with 0.2 M N-acetylglucosamine at pH 4.0. Further purification was achieved by gel-filtration on an AcA 54 column (Fig. 1b). The rice lectin was homogeneous according to several criteria: a single symmetrical peak was obtained by gel-filtration on AcA 54; it was superimposable on the agglutinating activity peak. Polyacrylamide gel electrophoresis at pH 4.3 revealed a single band. Immunodiffusion and immunoelectrophoresis using a rabbit antiserum raised against the crude lectin fraction also revealed a single precipitation line. The purification steps and yields for rice lectin are given in Table I.

Binding of [^{14}C] rice lectin to bacteria.

Table II indicates the data obtained with different nitrogen-fixing microorganisms isolated from the rhizospheres of rice and maize. Rice lectin specifically bound bacteria from the rhizosphere of rice. The *Beijerinckia V.* strain bound twice as much lectin as *Azospirillum lipoferum 4 B* strain. No significant binding was observed with the *Beijerinckia 39 J* or *Azospirillum B7C* strains from the rhizosphere of maize or with *Escherichia coli K 12* used as a control. The Scatchard plot for the interaction between rice lectin and *Beijerinckia V.* displayed a linear

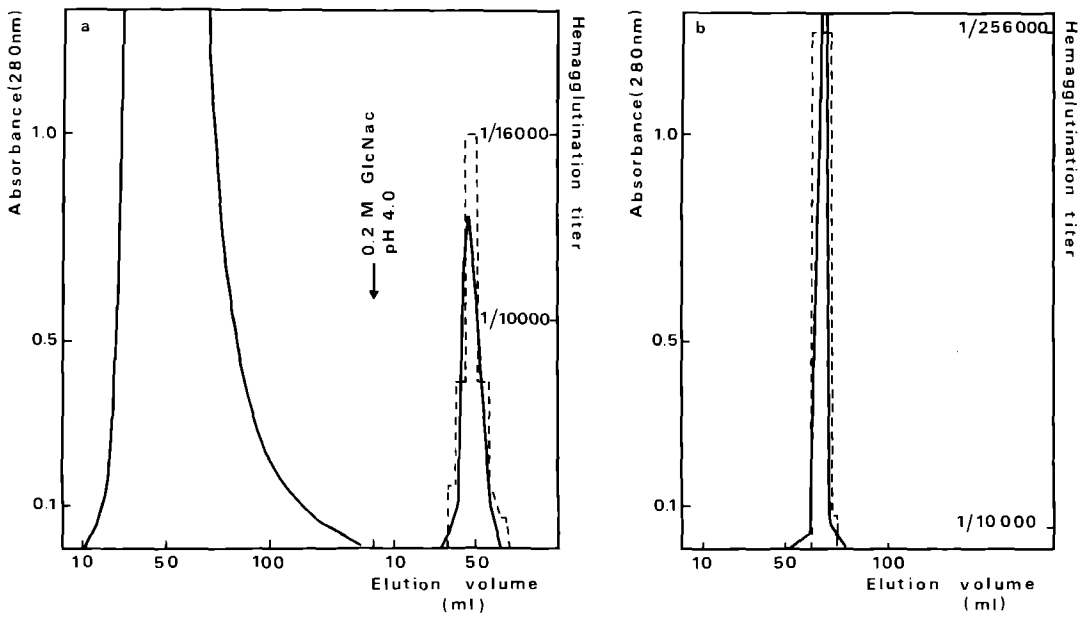

Fig. 1. Purification of the rice lectin :
a) affinity chromatography on agarose-p-aminophenyl-β-D-N-acetylglucosamine of crude lectin fraction (50 % ammonium sulfate precipitate).
b) gel-filtration on AcA 54 of the lectin fraction eluted from affinity chromatography column (Fig. 1a).

curve (Fig. 2), suggesting the existence of a single class of lectin binding sites on *Beijerinckia V.*. From this curve, the average number of bacteria-lectin binding sites was 1660 ± 150 per bacteria and the affinity constant at 20°C was (1.04 ± 0.30) x 10^7 M^{-1}.

TABLE I
Steps of purification of the rice embryo lectin
(*Oryza sativa*, var. Ballila 28)

Fraction	Total hemagglutinating activity (unit)	Specific hemagglutinating activity (unit/mg protein)	Activity yield %
Crude extracts from 100 g embryo	316 000	107	100
$SO_4(NH_4)_2$ precipitate			
P 50	324 700	422	103
P 100	2 350	4	<1
agarose-p-aminophenyl-β-D-N-acetylglucosamine	450 000	22 500	142
Gel-filtration on AcA 54	409 000	82 000	129

P 50 and P 100 fractions precipitate at 50 % and between 50-100 % ammonium sulfate saturation, respectively.
1 unit = minimum hemagglutinating dose against rabbit erythrocytes.

TABLE II

Amount of lectin bound to various strains of nitrogen-fixing bacteria from rice and maize rhizospheres and to enterobacteria *E. coli K 12*

Bacteria strains	Origin	Amount of lectin specifically bound to 10^8 bacteria (in dpm)
Beijerinckia V.	Rice rhizosphere	4467 ± 660
Azospirillum lipoferum 4 B	Rice rhizosphere	2230 ± 1227
Beijerinckia 39 J	Maize rhizosphere	< 400
Azospirillum lipoferum B7C	Maize rhizosphere	< 100
Escherichia coli K 12	Enterobacteria	< 300

DISCUSSION

This report describes a simple rapid procedure for rice lectin purification. Overall recovery and specific activity were high, compared to those reported earlier (12, 13). The procedure described by Takahashi

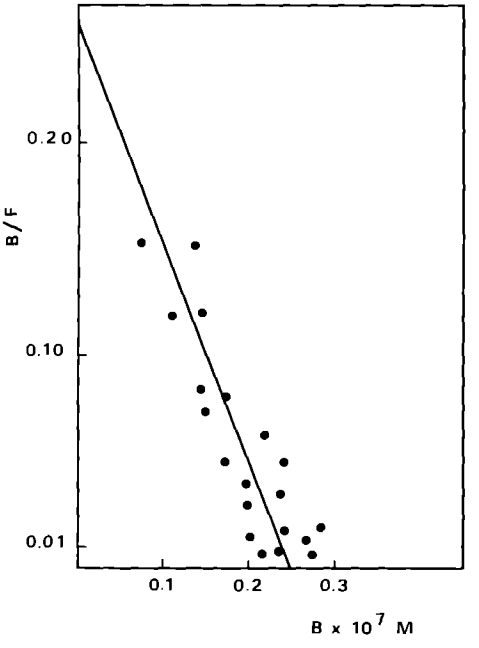

Fig. 2. Binding of the rice lectin to nitrogen-fixing bacteria *Beijerinckia V.* plotted according to Scatchard :
B : amount of bound lectin/10^8 cells.
F : amount of free lectin/10^8 cells.
The range of lectin concentration used was 3 to 100 µg/ml.

et al. (12) is rather time-consuming and gives a low yield. Tsuda (13) extracted the lectin activity at neutral pH and used ovomucoid-sepharose as an affinity adsorbent. In our experience, however, high lectin recovery requires extraction at low pH. Furthermore, the affinity of lectin for glycoproteins is at least 2 log units higher than its affinity for oligosaccharides. This could explain the incomplete elution from affinity column and low yields obtained by Tsuda (13). We observed here that rice lectin recovery was poor when the column was packed with chitin, a polymer of N-acetylglucosamine. Indravathamma and Seshadri (14) prepared the rice lectin by affinity chromatography on N-acetylglucosamine-Sepharose, but neither its homogeneity nor its hemagglutinating activity was reported. Consequently, it seems likely that the high lectin recovery and specific activity obtained in the present work were due to the extraction conditions and to the choice of affinity chromatography gel for the purification process.

Our preparation was 40-100 times more active (minimum hemagglutinating dose : 0.02 μg/ml) than that described by Tsuda (13). The differences observed in erythroagglutinating activities of various rice lectin preparations might be due to the varieties of rice tested and might also depend on the part of the plant used : seeds (12), bran (13) or, as in this work, the embryo.

There has been much speculation about the biological roles of lectins (5-7). For instance, it has been suggested that lectins participate in cell recognition processes. Many studies have been devoted to their possible role in the recognition between legumes and their bacterial nitrogen-fixing symbionts. In particular, soybean lectin (9, 10) and clover lectin (21) were shown to bind specifically and reversibly to carbohydrate components of infective strains of *Rhizobium japonicum* and *Rhizobium trifolii* bacteria respectively. The present results indicate that a gramineae lectin binds specifically nitrogen-fixing bacteria. Such binding, however, is restricted to microorganisms from the rhizosphere of rice. The rice lectin affinity for *Beijerinckia V.* is similar to that obtained for soybean lectin and *Rhizobium japonicum* (10). In contrast with the binding curve reported for soybean lectin, the curve for rice lectin and *Beijerinckia V.* was found to be monophasic. This suggests the existence of a single type of lectin receptor sites. The present demonstration of a specific high affinity binding of rice lectin for bacterial strains of the rhizosphere of rice strengthens the theory that lectins play an important role in the relation between plants and their symbiotic bacteria via a carbohydrate receptor.

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