HOST-SYMBIONT INTERACTIONS. I. THE LECTINS OF LEGUMES INTERACT WITH THE O-ANTIGEN-CONTAINING LIPOPOLYSACCHARIDES OF THEIR SYMBIONT RHIZOBIA[1]

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Summary:

A specific interaction between the O-antigen-containing lipopolysaccharides of Rhizobia and the lectins of their legume hosts has been demonstrated. The lectins have been purified from the seeds of four legumes and the lectins covalently attached to Agarose. The lipopolysaccharides were isolated from the four Rhizobial symbionts of the legumes. These four lipopolysaccharides were passed through the four lectin columns. In each case, the lipopolysaccharide from a Rhizobium interacts with the lectin column of its symbiont but not with the other lectin columns.

A major source of agriculturally important, biologically-fixed nitrogen results from the symbiosis between Rhizobia and legumes (1, 2). The formation of a successful symbiotic relationship is characterized by a high degree of host-symbiont selectivity (1, 2, 3). For example, one species of Rhizobia, R. japonicum, is able to form a symbiosis with soybeans but not with peas, while R. leguminosarum is a symbiont of peas but not of soybeans. Even within a single species of Rhizobia, there are numerous strains with varying abilities to form symbiotic relationships with the different cultivars of their symbiont hosts (1, 2, 3). While these general patterns of symbiont-host selectivity are recognized, the molecular basis for this selectivity is unknown. Several unsuccessful attempts have been made to involve in this specificity the exopolysaccharides secreted by the Rhizobia (4-9).

In considering the molecular basis of symbiont-host selectivity, it seemed attractive to ask whether a successful symbiosis requires an interaction between the O-antigens of the Rhizobia and the lectins of the legumes. Lectins are known to associate strongly with cell surface polysaccharides (see, for example, 10)

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and the O-antigens are exposed surface polysaccharides on gram-negative bacteria (11, 12). Hamblin and Kent have reported (13) that extracts of Red Kidney bean seeds contain at least one factor, presumably a lectin, capable of binding to R. phaseoli, the symbiont of Red Kidney beans. Addressing the question of the specificity of symbiosis, Bohlool and Schmidt (14) have presented evidence that soybean lectin is capable of binding to 22 of 25 infective strains of R. japonicum (the symbiont of soybeans), while soybean lectin does not bind to any of 23 other strains from 5 species of Rhizobia which do not nodulate soybeans.

Further evidence in support of the hypothesis that the lectins of legumes interact with the O-antigens of their symbiont Rhizobia has been obtained by immunological studies which demonstrate that the surface of Rhizobia has enough structural diversity to account for the observed host-symbiont selectivity (11, 15, 16). This structural diversity is very likely to reside in the O-antigens as these cell surface polysaccharides are not only structurally diverse but participate in determining the specificity of the interactions between gram-negative bacteria and other organisms (17-20). It seems reasonable that the molecules underlying those interactions already studied between gram-negative bacteria and other organisms are related to the molecules which underlie the selectivity of interactions exhibited by the Rhizobia and their legume hosts.

The present work demonstrates a specific interaction between the O-antigen-containing lipopolysaccharides of Rhizobia and the lectins of their legume hosts. These findings, determined by studying four species of Rhizobia and four species of legumes, are the first indications of a Rhizobial component involved in symbiont-host selection.

Materials and Methods:

Rhizobial Strains. Bacterial strains employed in this study were obtained through the courtesy of Dr. Joe Burton of The Nitragin Company and were: R. japonicum, strain 505; R. leguminosarum, strain 128-C-63; R. phaseoli, strain 127-K-24; and R. spp., strains 22A3 (from Jack bean) and 127-E-12 (from lima bean). Bacteria were grown at 25° using a yeast extract-mannitol medium (21). Cells were harvested in late log phase, frozen, stored on dry ice and kept at -20° until use.

Isolation of the O-antigen-containing Lipopolysaccharide. Lipopolysaccharide (LPS)* was isolated by the method of Westphal and Jann (22) with the

*LPS - lipopolysaccharide

following modifications. The cells (5-20 g) were thawed and washed 5 times (to remove a large proportion of the exopolysaccharide) by suspension in 10 ml of 0.155 M NaCl and centrifugation for 10 min at 12,350 x g. The pellet was suspended in 40 ml of 65° water and 40 ml of 65° phenol was added. The resulting slurry was stirred at 65° for 15 min, cooled on ice for 15 min, and centrifuged at 8,000 x g for 40 min at 6°. The water phase was set aside and the phenol phase was reheated to 65°, at which time 40 ml of 65° water was added and the above procedure was repeated. The two water extracts were combined and extracted 20 times with 25 ml of ether. The LPS was further purified by chromatography in water on Dowex 1X1 (Bio-Rad) which had been placed in the acetate form. The LPS (30 to 180 mg) was not retained by the column. The LPS was then freeze-dried and stored at 6°. The final purification consisted of chromatography of the LPS fraction (30 to 90 mg) on Bio-Gel A-1.5 (3.1 cm x 47 cm) in 20 mM imidazole·HCl, pH 7.0, containing 80 mM NaCl. The LPS was separated from the remaining exopolysaccharide by this procedure. The data of Figure 1 illustrate the separation of the LPS and exopolysaccharide of R. leguminosarum.

Chemical Modification of Lipopolysaccharide. Base-altered LPS was prepared by the method of Neter et al. (23). Lipopolysaccharide was freed of lipid by the method of Ryan and Conrad (24).

Isolation of the Lectins. Soybean lectin (SBA)[1] was isolated from seeds (10-20 g) of variety Chipewa. The purification procedure, up to the affinity chromatography, was that recommended by Lis and Sharon (25). The ammonium sulfate precipitated-soybean lectin was suspended in water, dialyzed overnight against water (containing 1 mM each of $MgCl_2$, $MnCl_2$, and $CaCl_2$), and centrifuged for 30 min at 37,000 x g. The supernatant solution, which contained the SBA, was applied to an Agarose column (1.3 cm x 4.5 cm) which had tetra-β-1,4-galactopyranosides covalently attached. This column was prepared by Dr. John Labavitch (26) by the method of Jeffrey et al. (27). The column, containing the SBA, was washed with 0.155 M NaCl until no more protein eluted. The retained SBA was eluted with 0.155 M NaCl containing 10 mM lactose. The purified SBA was dialyzed at 2° for three days against three changes of water containing 1 mM each of $MgCl_2$, $MnCl_2$, and $CaCl_2$. Finally the SBA was dialyzed for 3 hr against water without metals. The SBA was lyophilized and stored dry at -20°.

Austrian Winter pea lectin and Dark Red Kidney bean lectin (PHA)[2] were prepared as described below. Peas (10-20 g) or Red Kidney beans (10-20 g) were ground into a fine powder, extracted overnight with 1 mM sodium phosphate, pH 7.0, which was 0.155 M in NaCl. The suspension was centrifuged for 40 min at 48,200 x g. Ammonium sulfate (558 g/l) was added to the supernatant solution and, after stirring for 1 hr, the precipitated protein was collected by centrifugation for 20 min at 12,100 x g. The precipitated protein, containing the lectin, was dissolved in and dialyzed against two changes of phosphate-saline for 24 hr and then centrifuged for 15 min at 48,200 x g. The pea lectin preparation was applied to a Sephadex G-100 column (3.1 cm x 27.5) and washed with 0.155 M NaCl until no protein eluted from the column. The bound pea lectin was eluted with 0.155 M NaCl containing 50 mM D-glucose (28). The purified pea lectin was dialyzed and lyophilized as described for SBA. The PHA was purified on a thyroglobulin-Agarose column (15 ml containing 97 mg of thyroglobulin) by the method of Matsumoto and Osawa (29). The purified PHA was dialyzed and lyophilized as described for PHA.

Pea lectin, SBA, PHA, and Concanavalin A (Sigma Chemical Co.) were all coupled to Agarose gel as follows. Cyanogen bromide-activated Agarose gel (1.0 g, Pharmacia) was swollen and washed with 200 ml of 1 mM HCl followed by 10 ml of 0.2 M sodium citrate, pH 6.4. The lectin (5-10 mg), dissolved in 5 ml of the citrate buffer, and the washed gel were combined and mixed for 24 hr at 23°. The mixture was filtered and the gel washed with 5 ml of the pH 6.4 citrate buffer. Five mmoles of ethanolamine in 5 ml of a solution which was 0.1 M in

[1] SBA: soybean lectin

[2] PHA: Dark Red Kidney bean lectin

sodium bicarbonate and 0.5 M in NaCl, was added to the gel. After 3 hr at 23°, the gel was washed with 5 ml of 0.1 M sodium acetate, pH 3.4, which was 1.0 M in NaCl, and then with 5 ml of 0.1 M sodium bicarbonate. This 2-step washing cycle was repeated twice more. In the range of 50 to 90% of the added lectin was coupled to the gel in each case.

Chromatography of the Rhizobial Lipopolysaccharides on the Lectin-Agarose Column. The LPS from the four Rhizobial species was separately chromatographed at room temperature on each of the four lectin-Agarose columns (0.8 x 7.0 cm). The LPS sample (25-80 µg) was suspended in 0.1 to 0.4 ml of water and applied to the column which had been equilibrated in 20 mM imidazole·HCl, pH 7.0, containing 0.1 mM each of $MgCl_2$, $MnCl_2$, and $CaCl_2$. The LPS was washed into the column with 0.25 ml of column buffer, and the column flow was halted for 15 min. The column was then washed with 10 ml of the imidazole buffer at a flow rate of 5 to 10 ml/hr/cm^2. Any bound material was removed at acid pH (0.05 M glycine-HCl, pH 3.0, 0.5 M in NaCl). The presence of LPS was determined by assay for neutral sugars by a 0.1 ml micro-anthrone assay (30).

Protein was measured by the method of Lowry et al. (31) as modified by Eggstein and Kreutz (32) and by absorbance at 280 nm (33).

Results:

The characteristics of the isolated Rhizobial LPS and of the exopolysaccharide fractions were as follows. The LPS, which has an apparent molecular weight greater than 1.5×10^6 (by gel filtration), forms a viscous, opalescent solution in water. As expected (24), the dilute acetic acid treatment resulted in a soluble polysaccharide and a gummy, white precipitate which was soluble in chloroform. Sugar analysis of the intact LPS (work in progress) revealed dideoxyhexoses, aminohexoses, hexoses, and heptose, all sugars characteristic of LPS (11). The exopolysaccharide, which was separated by gel chromatography (Fig. 1) from the LPS, is of low molecular weight (in the range of 6,000) and consists largely of glucose with a small amount of galactose, matching the published composition (9, 10).

The results of the chromatography on a pea lectin-Agarose column of the LPS and the exopolysaccharides from the four Rhizobial species are shown in Figure 2. Of the four LPS samples, only a portion of the LPS from R. leguminosarum was retained by the column and even the carbohydrate in this LPS eluted from the column slowly; under the chromatographic conditions stated, 36% of the total anthrone positive carbohydrate in the R. leguminosarum was eluted at pH 3.0. These results, as well as those obtained under similar chromatographic conditions with the other three lectin columns, are summarized in Table I. As can be seen, only one LPS sample reacted with each lectin column.

TABLE I

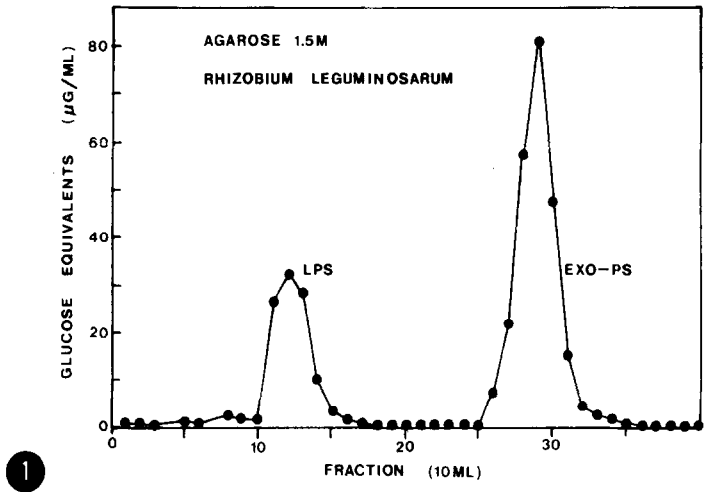
Interaction of Rhizobial lipopolysaccharide (LPS) and exopolysaccharide with immobilized lectins. The data represent the percentage of the anthrone positive lipopolysaccharides material which did not pass directly through the affinity column and which was eluted at pH 3.0 under the conditions described for Figure 2 and in the text.

	Source of immobilized lectin			
Rhizobial species and component	Soybean	Pea	Red Kidney bean	Jack bean
R. japonicum LPS	35	0	0	0
R. leguminosarum LPS	0	36	0	0
R. phaseoli LPS	0	0	5-23	0
R. spp. LPS	0	0	0	25
Exopolysaccharide from each of the above species	0	0	0	0

TABLE II

Comparison of the interaction of unmodified and modified R. phaseoli LPS with immobilized Red Kidney bean lectin (see Figure 2 and Table I for conditions of chromatography).

Treatment	Per cent of carbohydrate eluted at pH 3
Untreated LPS	14
Base-hydrolyzed LPS	16
Acetic acid-hydrolyzed LPS	13



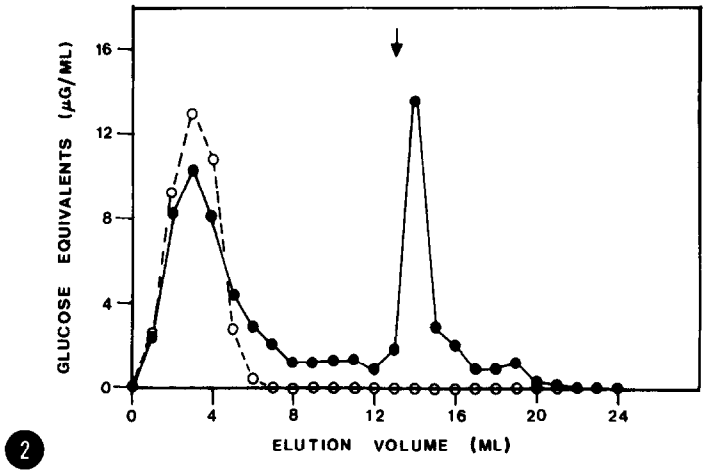


Figure 1. Gel filtration separation on Agarose A 1.5m of the lipopolysaccharide and exopolysaccharide obtained from R. leguminosarum. The details of the chromatography are in Materials and Methods. The carbohydrate content was estimated as glucose equivalents by the anthrone procedure (30).

Figure 2. Chromatography of the LPS from R. leguminosarum (●—●) on an Agarose-bound pea lectin column. The LPS was applied to the column in a pH 7.0 imidazole buffer. The column was washed at the point indicated by the arrow with a pH 3.0 glycine·HCl buffer. The LPS and exopolysaccharides from R. japonicum, R. phaseoli, and R. spp. and the exopolysaccharide from R. leguminosarum passed through the column in the pH 7.0 imidazole buffer (o---o). See the text for details. The glucose equivalent was estimated by the anthrone assay (30).

As noted in Table I, the interaction of the R. phaseoli LPS with the PHA column was somewhat variable, but a measurable interaction occurred consistently. In addition, Table I shows that the exopolysaccharides from the four Rhi-

zobial strains failed to interact with any of the lectin columns.

In order to investigate further the nature of the interaction between the lectin and LPS, LPS from R. phaseoli was treated (see preceding section) by two separate procedures designed to reduce its molecular weight. Base-treatment of the LPS results in de-esterification and reduction in the molecular weight of the LPS from about 10^6 to about 10^5 (23). The acetic acid treatment was designed to hydrolyze the ketosidic bonds and thereby separate the LPS into lipid and polysaccharide components. The O-antigen-containing polysaccharide has a molecular weight of about 20,000 (24). Both the base-treated LPS and the polysaccharide from the acetic acid-hydrolyzed LPS interacted with the PHA column to the same extent as the unmodified LPS (Table II).

Discussion:

The results reported in this paper strongly support the hypothesis that the high degree of specificity exhibited between a species of nitrogen-fixing Rhizobia and a legume is accounted for, at least in part, by the interaction of the Rhizobial somatic surface antigen (O-antigen) with the plant's lectin. Our approach has consisted of isolating, purifying, and immobilizing lectins from four species of legumes. The O-antigen-containing LPS, highly purified from four species of Rhizobia, were then chromatographed on the lectin-containing columns. In all cases, the Rhizobial LPS binds to that lectin which was isolated from the legume with which the Rhizobia is capable of entering into a symbiotic relationship. At no time does a Rhizobial LPS interact with a lectin isolated from a legume with which the Rhizobia cannot form a symbiotic relationship. Separation of the lipid and the O-antigen-containing polysaccharide portions of the LPS (Table II) provides evidence that it is the O-antigens which interact with the lectins. The fact that the maximum binding of a LPS of a Rhizobia to the lectin of its symbiont is 35% or less is not yet understood but is obviously of interest.

These findings are the first identification of a Rhizobial component (O-antigens) involved in the establishment of a successful symbiotic relation-

ship with leguminous plants. In agreement with two earlier publications (13, 14), we have shown that lectins are involved in this process. Further research will be necessary to show whether or not the interaction between lectins and O-antigens is required for the formation of a successful symbiosis.

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