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Accumulation of Soybean Lectin-Binding Polysaccharide During Growth of *Rhizobium japonicum* as Determined by Hemagglutination Inhibition Assay

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A hemagglutination inhibition assay was used to estimate the presence of soybean lectin-binding polysaccharide in whole culture, culture supernatant, and isolated exopolysaccharide of *Rhizobium japonicum* USDA 138. The occurrence of 0.1 to 0.2 μg of lectin-binding polysaccharide could be detected within 2 h with a 0.5-ml total sample. Lectin-binding polysaccharide was detected in all preparations during both exponential and stationary growth phases. The formation of lectin-binding polysaccharide was not, whereas that of total exopolysaccharide was, markedly affected by culture conditions.

Lectin receptors associated with *Rhizobium japonicum* cells have been described variously as exopolysaccharides (EPSs) (2-5, 10) and lipopolysaccharides (12). Specific interaction between *R. japonicum* EPS and soybean lectin (SBL) has been demonstrated by fluorescence microscopy (3, 4), Ouchterlony gel diffusion (10), radioassay (2), and electron microscopy (5, 10). Fluorescence and electron microscopy observations are limited to the study of cell-associated components, and gel diffusion lacks sensitivity. Radioassay is extremely sensitive, but the repeated washing required to remove excess unreacted radioactive label may also remove some EPS.

Resolution of the nature of the lectin-binding material of *R. japonicum* and the study of factors affecting its synthesis would be aided by improved methodology. In this report we describe a simple, rapid, and sensitive method based on hemagglutination inhibition for the assay of lectin-binding polysaccharide (LBP) in whole culture, culture supernatant, and isolated EPS of *R. japonicum*. Application of the hemagglutination inhibition assay (HIA) was made in a study of the production of LBP by a strain of *R. japonicum*.

MATERIALS AND METHODS

Culture. *R. japonicum* USDA 138 was used throughout. The stock culture was maintained on yeast extract-mannitol-salt (YMS) agar slants. Inoculum for YMS liquid culture was prepared by transfer to a YMS agar plate and incubation for 1 to 2 weeks at room temperature. The medium composition (in grams per liter) was as follows: mannitol, 10; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; and yeast extract (Difco certified), 1. Cultures were incubated on a rotary shaker at 25°C, and growth was followed by monitoring turbidity with a Klett-Summerson photoelectric colorimeter equipped with a no. 54 green filter.

EPS preparation. One- to two-week-old cultures of *R. japonicum* were centrifuged at 3,000 × *g* for 30 min. Cell-free supernatants were concentrated with a Diaflo membrane ultrafiltration device (Amicon Corp., Lexington, Mass.) with the XM300 membrane and desalted with distilled water. The concentrated preparations were lyophilized.

Purification of SBL. SBL was extracted from seed by the method of Liener and Pallansch (7). Crude lectin of the 30 to 70% ammonium sulfate-precipitable fraction was purified by affinity chromatography by the method of Allen and Johnson by using an acid-treated Sepharose 6B column (1). The hemagglutination titer of the final SBL preparation at a concentration of 100 μg/ml was 1,024 to 2,048.

Preparation of erythrocytes. Rabbit erythrocytes were trypsinized and washed with saline (0.9% NaCl) as described by Lis and Sharon (8). Trypsinized erythrocytes were then treated with 0.1% (vol/vol) glutaraldehyde (8% EM grade glutaraldehyde; Polysciences, Inc.) for 15 min and subsequently washed four times with saline by the method of Turner and Liener (11). A suspension of a 2.5% packed volume of erythrocytes in a hemagglutination buffer solution (9 g of NaCl, 1.27 g of Na_2HPO_4, 0.21 g of KH_2PO_4, 0.1 g of NaH_2PO_4 per liter of distilled water [pH 7.0]) was used with sodium azide of a 0.02% final concentration added as a preservative. The erythrocyte suspension so prepared was routinely used for 1 to 2 months without loss of sensitivity.

HIA. HIA was performed in a microtiter U plate (Dynatech Lab, Inc., Alexandria, Va.). Serial twofold dilutions of SBL (50 μl), starting at 100 μg/ml, were pipetted into microtiter plates so that wells in the same vertical row contained SBL solution of the same concentration and wells in the same horizontal row contained successively diluted SBL. EPS was added to the vertical row at 50 μl per well. Serial twofold dilutions of EPS were added so that wells of each

horizontal row contained EPS of the same concentration. After incubation at room temperature for 30 min, 100 μl of erythrocyte suspension of a 2.5% packed volume was added. A hemagglutination control with serially diluted SBL solution but without EPS was included in each assay. Heamgglutination titers were read after 2 h of incubation.

Anthrone carbohydrate determination. Carbohydrate content was determined by a modification of the anthrone colorimetric method described by Toennies and Kolb (9). A test tube containing 25 ml of 0.1% anthrone solution in 72% H_2SO_4 was chilled in an ice bath, and 0.5 ml of sample was layered on top of the reagent. Samples were mixed thoroughly, placed immediately in a boiling water bath for 10 min, and then cooled in a cold water bath. Optical densities were determined at 620 nm. Glucose solutions containing from 0 to 100 μg/0.5 ml were used as standards.

Ouchterlony gel diffusion. Ouchterlony gel diffusion was performed as described previously (10) in a petri dish containing 10 ml of 1% Noble agar in saline plus $MgCl_2$ to a final concentration of 10 mM.

RESULTS

Typical HIA results are shown in Fig. 1. Essentially linear dose responses were observed for both the cell-free, lyophilized EPS of *R. japonicum,* and the specific sugar hapten of SBL, *N*-acetyl-D-galactosamine. The minimal concentration of *N*-acetyl-D-galactosamine causing inhibition of hemagglutination was approximately 1 to 2 μg/ml. Hemagglutination was also inhibited proportionally by galactose with lower sensitivity. Glucose, mannitol, uninoculated culture medium, and 0.05 M phosphate buffer (pH 7.2) were tried as controls, and no inhibitory effect was observed.

As little as 0.2 μg of EPS per ml could be detected consistently by HIA. LBP, the active lectin-binding component of EPS, could be estimated from the relationships shown in Fig. 1 either in terms of EPS prepared as a standard or in terms of *N*-acetyl-D-galactosamine. In practice, for the isolation and purification of LBP and its assay in cultures, the amount of LBP was estimated in terms of the EPS standard assayed simultaneously with the test material. The relationships shown in Fig. 1 reveal the greater lectin-binding affinity of EPS relative to that of the sugar hapten. The presence of LBP was determined directly on samples from both the supernatant centrifuged at low speed and the whole, unfractionated culture during growth of *R. japonicum* (Fig. 2). LBP in the whole culture increased proportionally with growth during the exponential phase (up to 120 h) and increased slowly thereafter, indicating that the formation of LBP was associated mainly with active cell synthesis. LBP released into the supernatant first increased and then decreased slightly during the stationary phase. With incubation periods of up to 2 weeks, LBP in the supernatant remained high, but not as high as that observed at the end of the exponential phase.

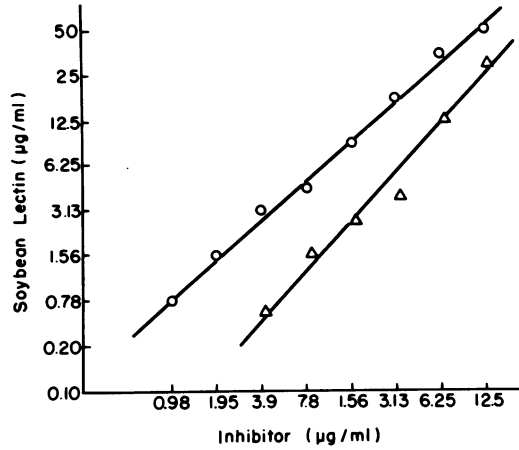

FIG. 1. *Hemagglutination inhibition by the EPS of R. japonicum USDA 138* (○) *and N-acetyl-D-galactosamine* (△). *The minimum concentration of SBL required to agglutinate erythrocytes in the presence of inhibitor was plotted versus the concentration of inhibitors on a base 2 log-log scale. The concentration of SBL at the base line (also Fig. 2, 5, and 6) is the minimum hemagglutination concentration in the absence of inhibitor.*

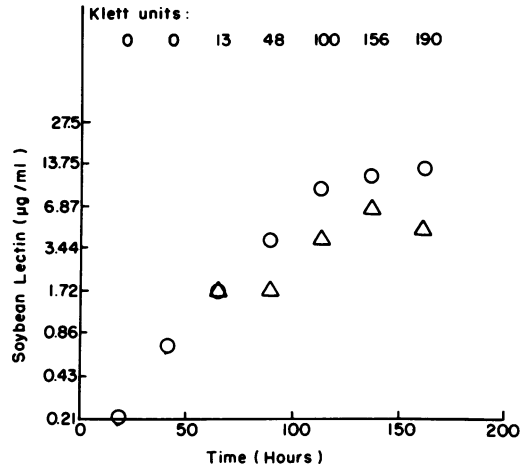

FIG. 2. *Change in hemagglutination inhibition activity during the growth of the R. japonicum culture. Hemagglutination inhibition activity, expressed as the concentration of SBL required for hemagglutination and plotted on a base 2 semilog scale, was determined on the whole unfractionated cultures* (○) *and the supernatants were centrifuged at low speed (3,000 × g for 30 min)* (△). *The optical density, measured with a Klett-Summerson photoelectric colorimeter, of each sample is indicated above each column.*

The removal of LBP from the supernatant by centrifugation at 3,000 × *g* for 30 min (Fig. 2) was associated with the sedimentation of an apparently high-molecular-weight EPS, especially during the stationary phase. This viscous white gel appeared after centrifugation as a white cell-gel complex layered above the pelleted cells. Volumetric measurements (Fig. 3A) showed that the white gel accumulated progressively throughout the growth cycle. Characteristics and lectin-binding properties of the white gel are included in another report (Tsien and Schmidt, submitted for publication).

Accumulation of LBP in culture supernatant was also estimated by semiquantitative Ouchterlony gel diffusion. Total non-dialyzable polysaccharide was determined by anthrone reagent. Samples were taken as numbered on the growth curve shown in Fig. 3B. Sample no. 1, taken at 24 h after inoculation, was not shown. Total non-dialyzable EPS increased logarithmically during the exponential phase and increased slowly thereafter during the stationary phase. After dialysis, samples were concentrated 10-fold and were diffused against SBL. Figure 4 shows a diffusion plate after 48 h of incubation. The center well contained SBL at 2 mg/ml. The result was evaluated semi-quantitatively by following the time sequence of precipitin band appearance, the relative intensities of precipitin bands, and the distance between bands and sample wells. The relative concentration of LBP was estimated to be in the order of 5 > 6 > 4 > 3 > 2. No precipitin band was observed between SBL and sample no. 1, even after prolonged incubation, indicating that LBP was either absent at the early stage of growth or present at a very low concentration. These results confirm those obtained by HIA.

The effect of the initial pH of the culture medium on LBP production was examined by growing *R. japonicum* in YMS medium adjusted to pH 6.5, 7.1, or 7.7. Slightly higher amounts of LBP were formed during growth at pH 7.7 than during growth at a lower initial pH. As averaged at 7 days from three experiments, production of LBP at pH 6.5 was only about 50% of that produced at pH 7.7. This was not a function of the growth rate since no significant differences occurred among the cultures with respect to turbidity at any of the sampling times. Also noticeable at the highest pH was increased production of white gel as measured volumetrically. Gel production at pH 7.7 was threefold that observed at pH 6.5.

Figure 5 shows the effect of aeration on LBP production by *R. japonicum* as demonstrated by varying the amount of culture medium in 125-

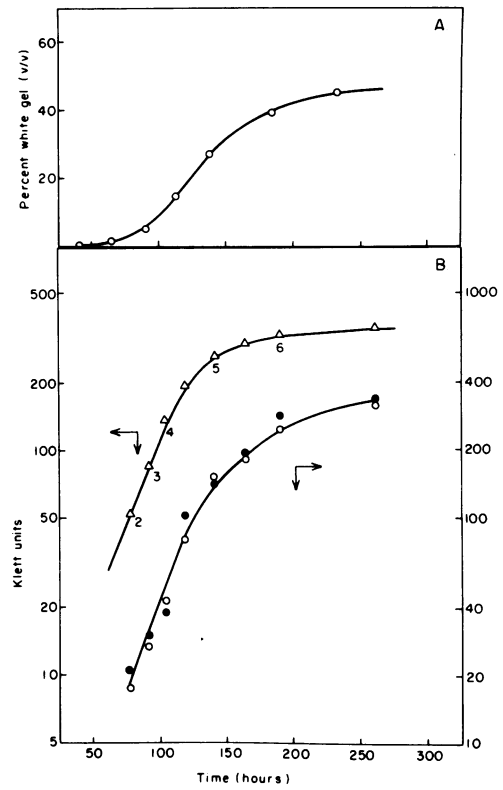

FIG. 3. *Typical growth curve of an R. japonicum culture (B) and accumulation of white gel sedimentable at low-speed centrifugation (A). The turbidity of the culture, in Klett units measured with a Klett-Summerson photoelectric colorimeter with a no. 54 filter, is plotted against the time of incubation* (△). *Carbohydrate in the culture supernatant, as measured by the anthrone colorimetric method and expressed as the glucose equivalent, is also plotted. Open and closed circles in (B) represent two different determinations. Percent white gel (A) measured volumetrically was plotted versus the time of incubation.*

ml conical flasks. LBP measured in the whole culture after 6 days was essentially the same irrespective of culture volume, but that in the supernatant increased with the increase in volume. The amount of the white gel indicated above each column in Fig. 5 and expressed as percent volume decreased with increased volume. The results indicate that LBP production was not affected by aeration, but that increased aeration promoted the formation of the high-molecular-weight white gel and also the association of LBP with that gel. Centrifugation to remove cells also sedimented the high-molecular-weight gel, taking some of the LBP with it as also shown in Fig. 2. The marked increase in the gel compared with relatively constant LBP sug-

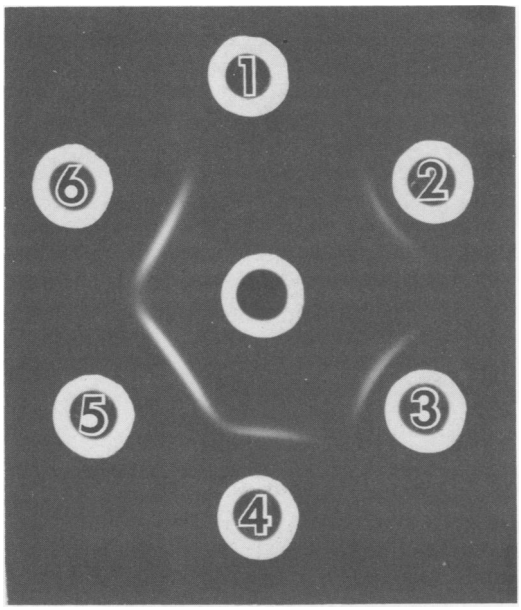

FIG. 4. *Ouchterlony gel diffusion of culture supernatants against SBL. The center well contained 0.1 ml of SBL at 2 mg/ml. Samples no. 1 to 6 were taken from the culture of R. japonicum during growth, as indicated by no. 2 to 6 in Fig. 3. Sample 1 was taken at 24 h after inoculation. Samples were concentrated 10-fold by dialysis against distilled water, lyophilized, and solubilized in phosphate buffer. Each well contained 0.1 ml of sample. The diffusion plate was photographed after incubation for 48 h.*

gests possible synthesis of one or more non-LBPs favored by increased aeration.

Addition of iron and calcium to YMS medium was accompanied in preliminary experiments by obviously enhanced production of the high-molecular-weight gel. Results of an experiment to confirm the effect of added calcium and iron on LBP, as measured by HIA, and on gel volume are shown in Fig. 6. Iron at the concentration shown in Fig. 6 was found previously to interfere with HIA; hence, all assays were preceded by dialysis of the samples. Under these growth conditions, LBP production was about the same or slightly lower than that normally assayed in standard YMS medium (Fig. 2, for example). A four- to fivefold increase in the white gel, however, was seen at the end of the exponential phase of growth (about 150 h). Up to 90% (vol/vol) of gel was observed after 2 weeks of incubation in this iron- and calcium-supplemented medium.

DISCUSSION

The work reported herein confirms our earlier observations (2–4, 10) and those of Bhuvaneswari et al. (2) and Cavert et al. (5) that the lectin-binding material of *R. japonicum* is found in the EPS complex. Controversy relative to the nature of this lectin-binding material can be resolved only by characterization of the isolated and purified active component. HIA, as commonly used for quantitative assay of antigen-antibody reactions (6), is a useful tool to assist in the isolation of the active lectin-binding material and the study of its synthesis. The method is simple, rapid, and sensitive, being capable of detecing LBP in the range of 1 μg or less. Due to its serial twofold dilution basis (base 2 logarithmic function), the assay can be applied over a wide range of concentrations, a feature which is useful for dealing with both crude culture filtrates and concentrates of the active material derived from them.

The active portion of EPS identified in this study as LBP largely by means of HIA was synthesized by cultures of *R. japonicum* USDA 138 throughout the growth cycle. LBP is not only associated with cells, as initially demonstrated by fluorescence microscopy (3), but may also diffuse away from cells to accumulate in the cell-free culture supernatant. Synthesis of LBP was found to be relatively constant not only with respect to growth phase, but also with respect to the factors of pH, aeration, and nutrition as examined briefly in this study.

At least one other polysaccharide, a viscous white gel material, was synthesized in addition

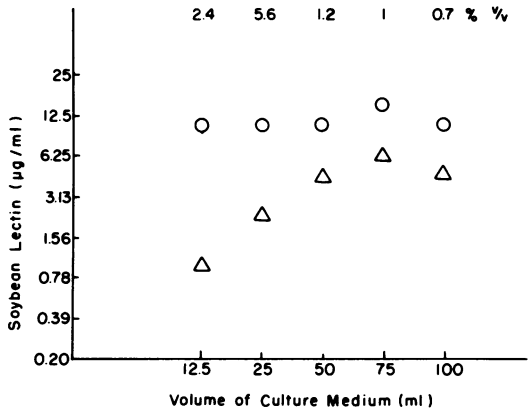

FIG. 5. *Effect of aeration on HIA and accumulation of LBP. An 8-h culture was distributed in different volumes as indicated and grown in 125-ml conical flasks for 6 days. Hemagglutination inhibition activities, expressed as the concentration of SBL required for hemagglutination on a base 2 semilog scale of whole cultures* (○) *and culture supernatants* (△), *are plotted against the volume. The volumetric measurement of white gel in percent is indicated above each column.*

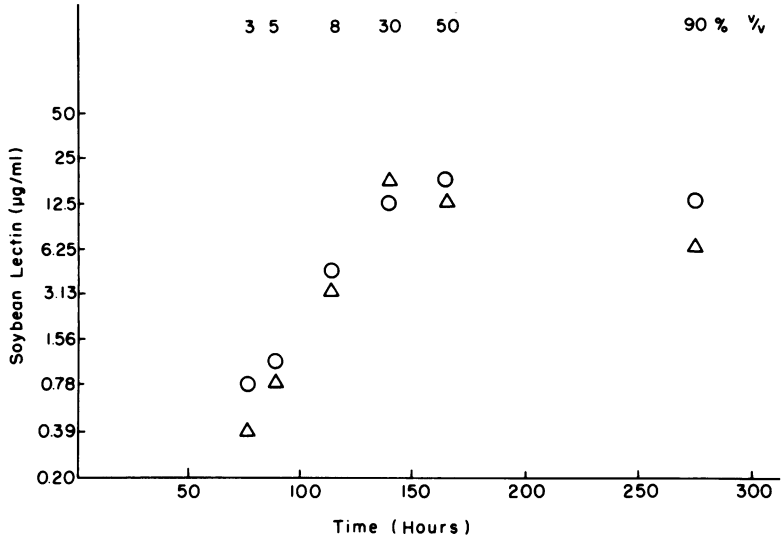

FIG. 6. *Effect of iron and calcium on the formation of LBP. Per liter of culture medium, 3.3 mg of $FeCl_3 \cdot 6H_2O$ and 2.66 mg of $CaCl \cdot 2H_2O$ were added. Hemagglutination inhibition activities, expressed as the concentration of SBL required for hemagglutination on a base 2 semilog scale, are plotted for whole cultures* (○) *and supernatants* (△). *Percent volume of white gel is indicated above each column.*

to LBP. Although obviously different from LBP, the gel may interact to modify LBP activity. Thus, when the gel was sedimented along with cells by centrifugation, some hemagglutination inhibition activity was lost from the supernatant. The interaction may be one of polymerization of LBP into the gel as the gel progressively increases with culture age or merely a physical trapping of LBP by the viscous gel. In contrast to LBP, synthesis of the gel was markedly affected by aeration and growth conditions.

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