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Interaction of *Pseudomonas solanacearum* Lipopolysaccharide and Extracellular Polysaccharide with Agglutinin from Potato Tubers

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In vitro binding assays were used to study the possible role of a cell wall agglutinin in the attachment to plant cell walls of avirulent strains of the wilt pathogen, *Pseudomonas solanacearum*. In a nitrocellulose filter assay, radioactively labeled lipopolysaccharide (LPS) from the virulent strain, K60, and the avirulent strain, B1, and extracellular polysaccharide (EPS) from K60 were bound quantitatively by the agglutinin extracted from Katahdin potato tubers. The LPS from B1 had significantly greater agglutinin-binding affinity than that from K60 but not after treatment with deoxycholate, which improved solubility. Highly purified chitotetraose did not inhibit binding of K60 LPS to agglutinin, but binding was inhibited by EPS as well as by diverse anionic polymers (DNA, dextran sulfate, xanthan). Binding of agglutinin to EPS and LPS was inhibited at ionic strengths greater than 0.03 and 0.15 M, respectively. It was concluded that electrostatic charge-charge interactions could account for binding of LPS and EPS to potato agglutinin.

Lectins, as well as macromolecules that do not have specific carbohydrate-binding properties, have been reported to agglutinate phytopathogenic bacteria and thus are of interest as possible determinants of host-pathogen interactions (25). Sequeira and Graham (27) reported that cells of avirulent strains of *Pseudomonas solanacearum* were strongly agglutinated by a hydroxyproline-rich glycoprotein extracted from potato tubers. Virulent cells, on the other hand, were weakly agglutinated. The agglutinin is a hydroxyproline-rich glycoprotein which is closely related to potato lectin, but unlike potato lectin, has very low hemagglutinating activity (14). Agglutination of avirulent *P. solanacearum* strains, which lack extracellular polysaccharide (EPS), could be prevented by adding microgram amounts of purified EPS to agglutinin-bacteria mixtures (27). Because lipopolysaccharide (LPS) from virulent strains precipitated when mixed with potato agglutinin, it was suggested that LPS could be the bacterial receptor for the agglutinin. Both agglutination of bacteria and precipitation of LPS were prevented by preincubating the agglutinin with oligomers of *N*-acetyl-D-glucosamine, obtained by hydrolysis of chitin (27). Graham and Sequeira (12) proposed that the agglutinin interacted specifically with sugars in the bacterial LPS. The ability of EPS to prevent bacterial agglutination was thought to result from specific competition for binding sites on the agglutinin.

Based on the facts that the agglutinin or antigenically similar proteins are present on tobacco cell walls (15) and avirulent cells are rapidly attached to these cell walls (26), it seemed likely that the agglutinin could be involved in recognition and immobilization of avirulent bacteria via LPS. Soluble EPS produced by virulent cells could prevent binding by occupying agglutinin binding sites in competition with LPS. Whatley et al. (34) reported that LPS from several avirulent strains of *P. solanacearum* lacked EPS as well as all or part of the O-antigen portion of LPS, raising the possibility that altered LPS may be a determinant in host recognition.

The interaction of potato agglutinin with LPS of virulent and avirulent *P. solanacearum* and with EPS of virulent strains is thus of interest because it may be involved in the host reaction to avirulent bacteria. In this paper, we report the use of a nitrocellulose filter membrane assay to study the interaction of *P. solanacearum* LPS and EPS with an agglutinin from potato tubers.

MATERIALS AND METHODS

Bacterial cultures. *P. solanacearum* isolates were obtained from A. Kelman (Department of Plant Pathology, University of Wisconsin-Madison). Bacteria were grown on modified TZC agar medium consisting of 10 g each of Bacto-Peptone (Difco) and glucose, 1 g of Casamino Acids (Difco), 0.05 g of triphenyl tetrazolium chloride, and 17 g of Bacto-Agar (Difco) per liter (13). Bacteria were stored in sterile distilled water in capped test tubes at room temperature.

Extraction and purification of bacterial polysaccharides. For batch extraction of *P. solanacearum* LPS and EPS, bacteria were grown in 2.4-liter Fernbach flasks containing 1 liter of CPG broth (10 g each of Bacto-Peptone and glucose and 1 g of Casamino Acids per liter). Inoculum for each batch consisted of a 10-ml culture started with a loopful of bacteria from a single colony from a 24-h culture grown on TZC agar overnight at 28°C. Batch cultures were incubated for 2 days on a rotary shaker at 28°C, and cells were harvested at late log or early stationary phase by centrifuging for 30 min at 10,000 × *g* at 4°C. Bacteria were washed twice by suspending pellets in phosphate-buffered saline (10 mM potassium phosphate–0.15 M NaCl [pH 7.2]) and centrifuging as before. Bacteria were resuspended in 50 to 100 volumes of distilled water, and LPS was extracted at 68°C by the phenol-water method (33). The pooled aqueous extracts from three extractions were dialyzed extensively against distilled water, lyophilized, redissolved in distilled water (ca. 10 mg/ml) and ultracentrifuged at 104,000 × *g* for 4 h. The pellet was suspended in distilled water and ultracentrifuged several times until suspensions in distilled water had an optical density of less than 0.01 at 260 and 280 nm. The purified LPS was suspended in distilled water, lyophilized, and stored at −20°C.

To purify EPS, 1 liter of batch culture filtrate was concentrated to a thick syrup by vacuum evaporation at 35°C, dialyzed extensively against distilled water, and brought to

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pH 7.0 by the addition of solid Tris. Magnesium chloride was added to a final concentration of 1 mM, followed by 25 μg/ml each of DNase (Worthington Diagnostics, Freehold, N.J.) and RNase A (Sigma Chemical Co., St. Louis, Mo.). After 4 h of incubation at 30°C, 50 μg of *Streptomyces griseus* protease (Sigma) per ml was added, and incubation was continued for an additional 4 h. The digest was heated to 100°C for 10 min, centrifuged for 10 min at 11,000 × *g*, and the supernatant was removed and extracted once with hot aqueous phenol (33). The aqueous layer was dialyzed, and trace amounts of LPS were removed by ultracentrifugation. Purified EPS preparations were assayed for protein by the method of Bradford (5) and for 2-keto-3-deoxyoctulosonic acid by the thiobarbiturate method (7). EPS was also analyzed for phosphorous (3), uronic acids (4), and pyruvate (28). EPS and LPS were lyophilized and stored at −20°C.

Radiolabeled LPS and EPS. To obtain ^{14}C-labeled LPS and EPS of high specific activity, a mineral salts-based medium containing 1.3 g of $(NH_4)_2SO_4$, 0.20 g of $MgSO_4 \cdot 7H_2O$, 0.92 mg of $MnSO_4 \cdot H_2O$, 3.3 mg of $ZnSO_4 \cdot 7H_2O$, 2.4 mg of $FeCl_3 \cdot 6H_2O$, 2 g of $CaCO_3$, and 0.33 g of Casamino Acids per liter in 0.01 M phosphate buffer (pH 7.0) was employed. The medium was autoclaved, and then 20 μCi of D-[*U*-^{14}C]glucose (specific activity, 240 mCi/mmol) (ICN, Irvine, Calif.) and 2.5 mg of unlabeled, filter-sterilized glucose per ml was added. The medium was dispensed in 10- to 25-ml portions in Erlenmeyer flasks, a loopful of a bacterial suspension from 48-h cultures of *P. solanacearum* grown on TZC was added to each flask, and then the flasks were incubated at 28°C in a shaker for 36 h. LPS and EPS were extracted and purified as described above and stored at 4°C in lyophilized form or as filter-sterilized suspensions in distilled water.

The purity of ^{14}C-LPS preparations was checked by thin-layer chromatography of acid hydrolysates (32). ^{14}C-LPS (ca. 10 μg) was hydrolyzed with 1 N sulfuric acid for 4 h at 100°C. The hydrolysate was neutralized with $BaCO_3$, and the precipitate was removed by low-speed centrifugation. The supernatant solution was concentrated by lyophilization and spotted on Silica Gel G (E. Merck AG, Darmstadt, Federal Republic of Germany) plates along with sugar standards (1%) in distilled water. Plates were developed twice with methyl ethyl ketone-acetic acid-methanol (6:2:2) and sprayed with aniline-diphenylamine-phosphate (30) to visualize the sugar standards. Then 2.5-mm strips were scraped from the gel and suspended in toluene-Triton scintillation fluid, and radioactivity was determined with a scintillation counter. The R_f values of peaks of radioactivity were compared with the values obtained with sugar standards that had been chromatographed in parallel.

Chitin oligomer preparation. Chitin oligomers were prepared by a modification of the methods of Rupley (24). Crab shell chitin (100 g; Sigma) was dissolved slowly in 1 liter of concentrated HCl at 20°C and incubated with stirring for 4 h. The solution was neutralized with 12 N NaOH, and the neutralized suspension was filtered through Whatman no. 2 filter paper. The filtrate was eluted through an activated charcoal-Celite (1:1) pad and washed extensively with distilled water until the conductance, as measured with a conductivity bridge (Industrial Instruments, Cedar Grove, N.J.), was reduced to that of distilled water. Then chitin oligomers were eluted from the charcoal-Celite with 30% ethanol, and the eluate was evaporated to dryness by vacuum evaporation at 35°C. Twenty milligrams of the crude chitin oligomers dissolved in 2 ml of 0.1 M ammonium bicarbonate buffer, pH 8.0, were applied to a Bio-Gel P-2 column (2.5 by 60 cm) equilibrated with the same buffer. Fractions (2 ml) were collected and assayed for reducing sugars by the ferricyanide method (21), with *N*-acetyl-D-glucosamine as a standard. Fractions that corresponded to peaks of reducing activity were lyophilized, and the degree of polymerization was estimated by determining the ratio of micrograms (dry weight) to micrograms (reducing equivalents). Purified oligomer fractions were assayed for free amino groups by incubating a water suspension (10 mg/ml) with an equal volume of 3% ninhydrin reagent in ethanol (Sigma) at 110°C for 10 min (20). Standards included *N*-acetyl-D-glucosamine and D-glucosamine (Sigma). Identity of oligomers was further checked by thin-layer chromatography on Silica Gel G with methanol-butanol-water (30:30:10) (30). Plates were sprayed with 4% silver nitrate in acetonitrile-ethanol (9:1) and 10 N NaOH–ethanol (1:10).

Fractions containing chitin hydrolysates with degrees of polymerization of 2 to 6 and the higher-molecular-weight void fraction were dissolved in 0.01 M citrate-phosphate buffer (CPB) (pH 5.5) and tested for their ability to inhibit LPS-agglutinin binding in the nitrocellulose filter assay. The void fraction was dialyzed with distilled water, and a portion of the dialysate was applied to a DEAE-Sephadex column (3 ml, bed volume) in 0.02 M Tris (pH 7.2). The excluded fraction was collected, the column was washed with 0.2 M NaCl, and the eluted material was recovered. A second portion of the dialysate was applied to a carboxymethyl-Sephadex column in 0.02 M phosphate buffer (pH 7.2), and the excluded fraction was collected. All fractions were dialyzed into CPB and tested for inhibition of agglutinin-LPS binding.

In certain experiments, pure chitotriose (Sigma) and chitotetraose (a gift of M. Schindler) were also used in hapten inhibition tests.

Purification and labeling of potato agglutinin. Purified potato agglutinin, extracted from potato tubers (cultivar Katahdin) was a gift of J. Leach and M. Cantrell. The agglutinin was stored at 4°C in 0.15 M sodium acetate and 0.15 M NaCl (pH 3.6) (14, 17).

^{3}H-labeled Katahdin agglutinin was prepared by a modification of the procedure of Lotan et al. (16). Purified agglutinin (1 mg) in 0.5 ml of 0.1 M sodium acetate (pH 4.5) was mixed with 1 ml of 0.05 M sodium metaperiodate in the same buffer at 0°C. After 45 min of incubation in the dark, 100 μl of ethylene glycol was added to reduce excess periodate, the mixture was dialyzed in 0.1 M potassium phosphate (pH 8), and then 1.4 mCi of [^{3}H]sodium borohydride (ICN; 260 mCi/mmol) in dimethyl sulfoxide was added with stirring. After 10 h of incubation, the pH of the mixture was lowered to 4.0 with 0.1 M acetic acid, and then the mixture was dialyzed against distilled water and concentrated to 0.7 ml by ultrafiltration. The labeled agglutinin was then chromatographed on a Sepharose 4B-Cl column (1.5 by 27 cm), and fractions from the peak of ^{3}H activity at $V_e = 0.8$ (corresponding to that of untreated agglutinin) were pooled and concentrated by ultrafiltration. The preparation was filter sterilized and stored at 4°C.

Agglutination and precipitation assays. Bacterial agglutination was assayed as described by Sequeira and Graham (27). Equal volumes (10 μl) of bacterial suspensions (optical density at 600 nm of 0.85) and serial $\log_2$ dilutions of agglutinin in CPB were mixed as drops on polystyrene petri plates. After 12 h at 21°C, the suspensions were rated for agglutination on a scale of 0 to 4. For precipitation assays, bacterial polysaccharides were suspended in CPB at 0.1 mg/ml, and 20-μl samples were mixed with 20 μl of serial $\log_2$

dilutions of agglutinin in the same buffer. Reactions were rated after 60 min of incubation on the same scale as for bacterial agglutination.

For hemagglutination assays, rabbit erythrocytes were suspended in 0.1% trypsin (Calbiochem-Behring Corp., San Diego, Calif.) for 1 h at 37°C, washed with phosphate-buffered saline (0.12 M NaCl, 0.01 M Na_2HPO_4, 0.003 M K_2HPO_4 [pH 7.2]), and then fixed with 0.1% (vol/vol) glutaraldehyde for 10 min at 24°C. Cells were suspended in phosphate-buffered saline and adjusted to an optical density at 600 nm of 0.8. Hemagglutination assays were done as described for bacterial agglutination. Potato lectin was obtained from P & L Biochemicals, Milwaukee, Wisc.

Nitrocellulose filter membrane binding assay. Dilute ^{14}C-LPS suspensions in CPB were homogenized for 1 min at 2°C with a probe sonicator set at 30% maximum power. In other experiments, soluble preparations of radiolabeled LPS were obtained by treating with sodium deoxycholate by the procedure of Ribi et al. (22).

For binding assays, samples of an appropriate dilution of agglutinin and labeled polysaccharide were mixed (total volume, 200 μl) and incubated in plastic microtiter wells for 15 min at 21°C. Each incubation mixture was transferred onto a prewetted nitrocellulose filter membrane (diameter, 13 mm; pore size, 5 μm; type SMWP; Millipore Corp., Bedford, Mass.) placed on a filter support connected to a vacuum manifold. Filter supports consisted of Swinnex filter holders (Millipore) with the tops removed. Samples were filtered by applying vacuum to the manifold and were then washed by rinsing with 300 μl of CPB.

In some experiments, other proteins and glycoproteins were tested for LPS-binding ability in the nitrocellulose filter assay. Ten micrograms of protein and 0.8 μg of K60 ^{14}C-LPS were mixed in 200 μl of CPB, and assays were carried out as before.

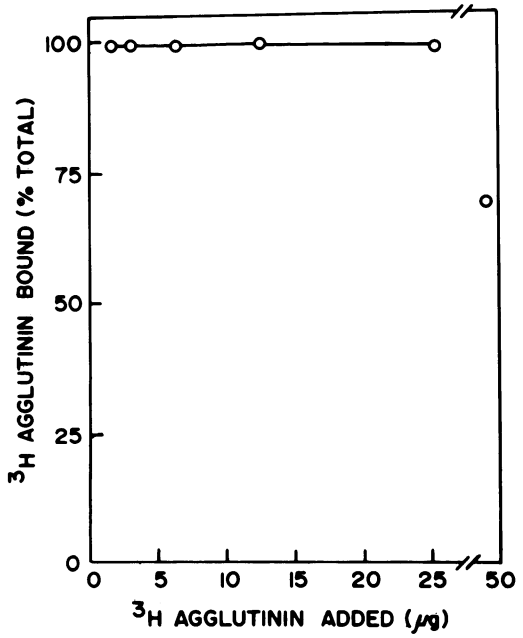

FIG. 1. Binding capacity of nitrocellulose filter membranes for potato agglutinin. From 0.04 to 50 μg of ^3H-agglutinin (3,300 dpm/μg) in CPB (200 μl) was filtered through 13-mm Millipore SMWP nitrocellulose filter membranes as described in the text. Radioactivity retained on filters and recovered in wash fluid was converted to micrograms of ^3H-agglutinin.

Membranes were dried at 120°C for 5 min and placed in 5 ml of scintillation fluid. In some experiments, both the initial effluent and the washings were collected directly into scintillation vials positioned below each filter support, and radioactivity was determined as before. Radioactivity of assay samples was determined with a Packard PRIAS liquid scintillation counter (Packard Instrument Co., Inc., Downer's Grove, Ill.). Nitrocellulose membranes were counted in toluene-based fluor, and aqueous samples were counted in toluene fluor plus Triton X-100.

RESULTS

Characteristics of purified radiolabeled materials. The specific radioactivity of purified ^{14}C-labeled LPS preparations, on a dry weight basis, was ca. 900 and 2,300 dpm/μg for B1 LPS and K60 LPS, respectively.

From 1 to 3% of the total label added was recovered in LPS. Analysis of acid hydrolysates of these LPS preparations by thin-layer chromatography showed no significant amount of label comigrating with ribose (R_f = 0.70) or galactosamine (R_f = 0.62). On this basis, ^{14}C-LPS preparations appeared relatively free of nucleic acid and EPS. Galactosamine is the major sugar component of *P. solanacearum* EPS (8) but not of LPS. K60 ^{14}C-EPS had a specific radioactivity of ca. 1,800 dpm/μg.

The ^3H-labeled Katahdin agglutinin had a specific radioactivity of ca. 3,000 dpm/μg. ^3H-labeled agglutinin was not altered in its ability to bind ^{14}C-LPS and ^{14}C-EPS on nitrocellulose filter membranes (data not shown).

Retention of ^3H-agglutinin and ^{14}C-labeled EPS and LPS on nitrocellulose filter membranes. The proportion of ^3H-agglutinin retained on 13-mm SMWP filter membranes as a function of concentration of agglutinin was determined (Fig. 1). From 98 to 100% of the ^3H was retained on the membrane in the range of 0.05 to 25 μg of ^3H-agglutinin (Fig. 1). All subsequent binding experiments were carried out in the range of 0.1 to 10 μg of agglutinin per membrane, a level well below saturation. Other experiments demonstrated that retention of ^3H-agglutinin on membranes was not affected by pH (3.0 to 10.0), NaCl (up to 2 M), or chitin oligomers (data not shown).

^{14}C-labeled EPS was retained at a very low level (ca. 5%) on filter membranes, and this background binding was independent of the amount of EPS applied to the membrane. Binding of B1 and K60 ^{14}C-LPS to filter membranes also was low but varied between preparations (5 to 15%) and was higher for deoxycholate-treated preparations (up to 20%). When B1 or K60 ^{14}C-LPS was sonicated, however, the background binding was reduced to ca. 10%; therefore, LPS preparations were routinely sonicated before use.

Binding of ^{14}C-EPS and ^{14}C-LPS to potato agglutinin on membranes. To determine the concentration of agglutinin required to bind the maximum amount of ^{14}C-polysaccharide, a constant amount of ^{14}C-EPS or ^{14}C-LPS was incubated with increasing amounts of agglutinin. The amount of label retained on filters increased with increasing agglutinin until essentially 100% of the ^{14}C-label was retained (data not shown). Concentrations of agglutinin and polysaccharide were chosen to correspond to the linear portion of the curve in which from 50 to 70% of the polysaccharide was bound.

In other experiments, the amount of ^{14}C-polysaccharide was varied in the presence of a constant amount of agglutinin. The dose-response curves for B1 and K60 LPS were dissimilar; considerably more B1 than K60 LPS was bound per microgram of agglutinin. After deoxycholate treatment

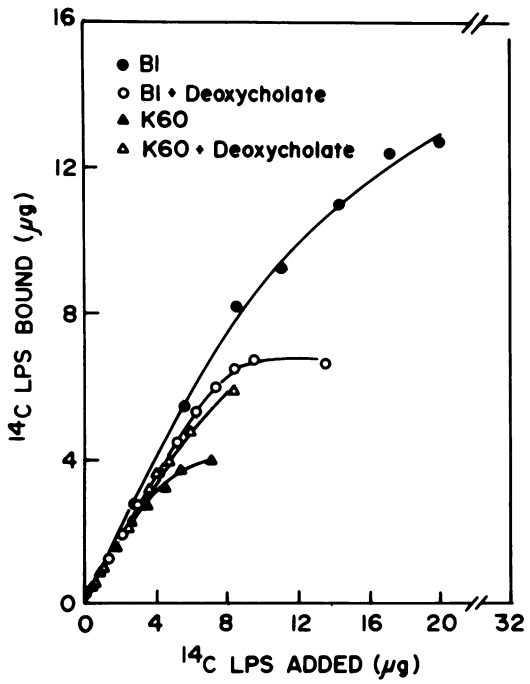

FIG. 2. Binding of untreated and deoxycholate-treated K60 and B1 ^{14}C-LPS to potato agglutinin as a function of LPS concentration. Incubation mixtures (200 μl) contained 8 μg of agglutinin and from 0.4 to 8.0 μg of K60 ^{14}C-LPS or from 1 to 32 μg of B1 ^{14}C-LPS in CPB. Values have been corrected for background binding of LPS to nitrocellulose membranes.

of LPS, however, curves for B1 and K60 LPS reached apparent saturation at similar levels (Fig. 2).

Effect of chitin oligomer fractions on LPS and EPS binding to agglutinin. Oligomer fractions did not have a significant reaction with ninhydrin, indicating that deacylation had not occurred. Thin-layer chromatography of the P-2 fractions confirmed that oligomers up through chitotetraose were present in these fractions (data not shown).

When chitin oligomer fractions were tested in LPS-agglutinin binding assays, only the void peak (fraction 0) had significant inhibitory activity. Also, only this fraction prevented binding of K60 EPS to the agglutinin. Approximately 25 and 0.5 μg of fraction 0 gave 50% inhibition of binding of 2 μg of K60 ^{14}C-LPS and 0.5 μg of ^{14}C-EPS, respectively (Fig. 3).

When fraction 0 was dialyzed with CPB, 46% of the dry weight remained, and the dialysate still inhibited LPS binding to the agglutinin. When chitin hydrolysate was eluted through DEAE-Sephadex, however, the material that was retained and then eluted with 0.5 M NaCl (40% of the total dry weight) contained all of the inhibitory activity of fraction 0. We have not identified the active component of fraction 0.

Purified chitotetraose, which is a very effective hapten of potato lectin (1), did not inhibit agglutinin-LPS binding at concentrations as high as 3 mM (Table 1). Chitotetraose and chitotriose (from Sigma) at 3 mM also did not prevent agglutination of B1 bacteria by potato agglutinin (data not shown). That the chitotetraose was active was shown by its ability to inhibit the agglutination of rabbit erythrocytes by wheat germ agglutinin and potato lectin, both specific for *N*-acetylglucosamine oligomers (data not shown).

Inhibition of ^{14}C-LPS binding by EPS and other macromolecules. Unlabeled EPS from K60 was an effective inhibitor of the binding of K60 ^{14}C-LPS to potato agglutinin (Fig. 4). Approximately 17 μg of K60 EPS per 200 μl of incubation mixture resulted in 50% inhibition. Anionic polymers (xanthan, calf thymus DNA, and dextran sulfate) also inhibited LPS binding (50% inhibition) at concentrations ranging from 0.5 to 10 μg/200 μl of incubation mixture (Fig. 4). Dextran, an uncharged polymer, had no effect at 100 μg. The charged polymers were each precipitated when agglutinin was added in microtiter wells (data not shown).

Influence of ionic strength and pH on binding of LPS and EPS to potato agglutinin. K60 EPS was bound optimally over a very narrow range of ionic strength (0 to 0.03 M), whereas binding of K60 LPS was essentially unchanged up to 0.12 M (Fig. 5). When LPS and agglutinin were mixed in 0.01 M CPB, filtered onto nitrocellulose membranes, and then washed with increasing concentrations of NaCl, no significant reduction in binding was detected, even at concentrations as high as 1 M NaCl (Fig. 5). Both K60 LPS and EPS interacted with Katahdin agglutinin over a pH range of 4 to 8 (data not shown).

Binding of K60 LPS to various proteins and glycoproteins. Other proteins and glycoproteins were tested for their ability to bind LPS and EPS on nitrocellulose membrane filters and in the precipitin assay (Table 2). Two basic proteins, polylysine (Sigma) and corn histone (a gift of J. Kermickle), bound significant amounts of K60 LPS, but wheat germ agglutinin and soybean agglutinin bound low amounts of LPS. Another basic protein, lysozyme (pI = 11.35), and an acidic protein, bovine serum albumin (pI = 4.7), bound very little LPS.

DISCUSSION

The results demonstrated the usefulness of the nitrocellulose membrane assay as a means to measure protein-polysaccharide interactions in vitro. Potato agglutinin was quantitatively bound to membranes at levels below 10 μg per membrane, whereas only small amounts of LPS and EPS

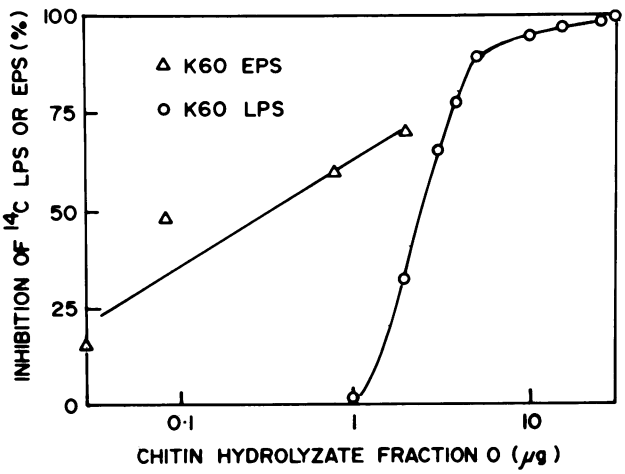

FIG. 3. Effect of chitin hydrolysate fraction 0 on binding of K60 ^{14}C-LPS and ^{14}C-EPS to potato agglutinin. For LPS inhibition studies, agglutinin (1.5 μg) and fraction 0 (10 to 300 μg) were incubated in CPB (200 μl) at 21°C for 15 min, and then 2 μg of ^{14}C-LPS was added. For EPS inhibition studies, agglutinin (0.6 μg) and from 0.25 to 25 μg of fraction 0 were incubated as described above, and then 0.5 μg of K60 ^{14}C-EPS in 10 μl of CPB was added. After 15 min, mixtures were washed through nitrocellulose filter membranes. Points represent the mean of two samples in a single experiment (EPS) and two experiment (LPS).

were retained. When preincubated with agglutinin, however, LPS from both avirulent and virulent strains of *P. solanacearum* was retained on nitrocellulose membranes. Although differences in the level of binding of B1 and K60 LPS could be detected, these differences were largely abolished when LPS was treated with deoxycholate before assay. Deoxycholate treatment improves the solubility of LPS that lacks O-antigen by allowing the molecules to reaggregate slowly to form uniform micelles when deoxycholate is removed (22). Thus, deoxycholate may have reduced the size of the B1 LPS micelles and thus correspondingly reduced the amount of weight bound per microgram of agglutinin. The increase in background binding of LPS to nitrocellulose after deoxycholate treatment is not understood but could be due to a change in the number or distribution of sites on micelles that interact with nitrocellulose.

In an earlier report (27), binding of the agglutinin to bacteria and to LPS in microtiter wells was inhibited by chitin oligomers. We could not demonstrate inhibition of agglutinin binding of LPS when highly purified potato agglutinin and chitotetraose were used. Although a high-molecular-weight fraction from hydrolyzed chitin was effective in preventing agglutinin-LPS binding, the active component was nondialyzable and contained anionic groups and thus may not be a typical saccharide hapten.

Some of the inconsistencies between the hapten reversibility data we obtained and earlier work may be explained by the recent demonstration that the bacterial potato agglutinin is distinct both in biological activity and in physicochemical properties from the potato lectin described by Allen and Neuberger (1). Highly purified potato agglutinin agglutinated rabbit erythrocytes very weakly but retained B1-agglutinating ability. A purified preparation of potato lectin, on the other hand, showed little or no B1-agglutinating ability but agglutinated rabbit erythrocytes (15). The two glycoproteins are rich in hydroxyproline but differ in total amino acid composition and molecular weight. Possibly, earlier preparations of bacterial agglutinin contained mixtures of the two proteins. Also, it is possible that earlier chitin oligomer preparations contained an anionic component, similar to fraction 0, which was responsible for the observed inhibition.

Several lines of evidence support a charge-charge interaction in the binding of potato agglutinin to bacterial polysaccharides. The inhibition of LPS-agglutinin binding by several polyanionic but not by neutral molecules (Fig. 5) is indicative of an interaction dependent on electrostatic forces. The inhibition of LPS binding at moderate to high ionic strength is also consistent with a charge model. EPS binding was more sensitive to salt than that of LPS, and this may be due to a lower density of charged groups on the EPS molecule.

TABLE 1. Effect of pure chitotetraose[a] on binding of K60 ^{14}C-LPS to potato agglutinin

Concn of chitotetraose		^{14}C-LPS bound to potato agglutinin[b]	
mg/ml	mM	dpm[c]	% Control
0.00	0.00	901	100
0.05	0.06	884	89.1
0.25	0.30	903	100.0
2.5	3.0	924	102.0

[a] Obtained from M. Schindler.
[b] 0.2 μg per sample.
[c] Mean of two samples.

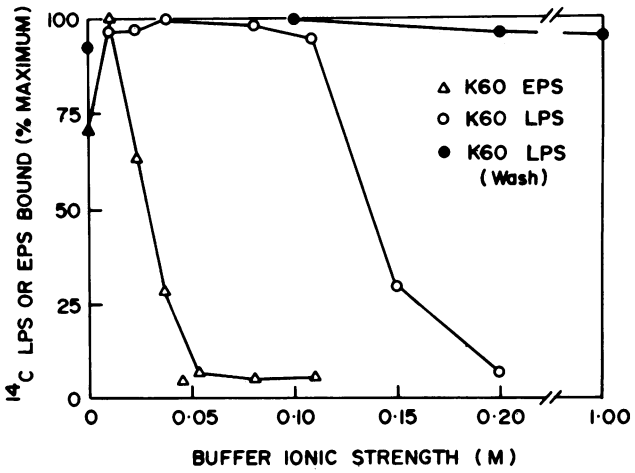

FIG. 4. Binding of K60 ^{14}C-LPS and ^{14}C-EPS to potato agglutinin as a function of buffer ionic strength. For LPS binding studies, agglutinin (0.5 μg) was mixed with 2 μg of K60 ^{14}C-LPS in CPB (200 μl) containing increasing amounts of NaCl. After 15 min of incubation, mixtures were washed through nitrocellulose filter membranes with an additional 0.5 ml of the same buffer. In a parallel experiment (solid circle), LPS and agglutinin were incubated in CPB without added salt but after filtration onto filter membranes received a wash consisting of 0.5 ml of CPB plus increasing amounts of NaCl. For EPS binding studies, agglutinin (2 μg) was mixed with 2 μg of K60 ^{14}C-EPS in CPB (200 μl) containing increasing amounts of NaCl. Incubation and assay conditions were as described above for LPS. Amount of polysaccharide bound, expressed as a percentage of the maximum amount bound, is plotted against ionic strength of the incubation or wash buffer. Points represent the mean of two or three samples in a single experiment.

The agglutinin is a highly basic molecule, with a pI of >11.0 (14). The pH range for binding of LPS and EPS to the agglutinin (3 to 8) is not inconsistent with an electrostatic model, given the extremely high pI of the agglutinin. An examination of the LPS-binding ability of various proteins (Table 2) suggests that some, but not all, basic proteins can interact with K60 LPS. The lack of binding of LPS by

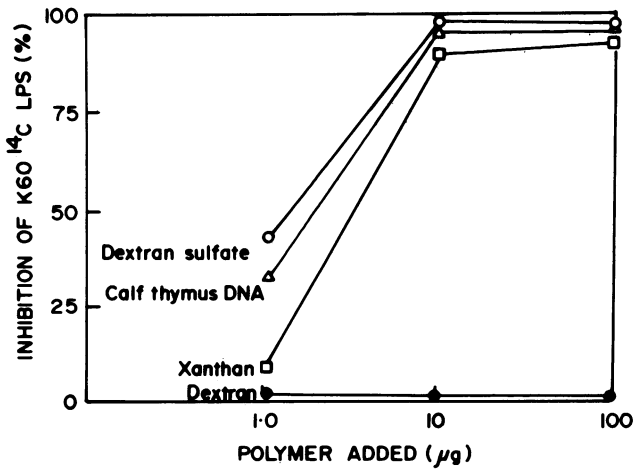

FIG. 5. Effect of charged and uncharged polymers on binding of K60 ^{14}C-LPS to potato agglutinin. Agglutinin (2 μg) and various amounts of polymers were incubated 15 min in CPB (190 μl), and then 2 μg of ^{14}C-LPS in 10 μl of CPB were added. After 15 min, mixtures were washed through nitrocellulose membrane filters. Points represent the mean of two samples in a single experiment.

TABLE 2. Binding of K60 LPS to various proteins and glycoproteins

Protein[a]	pI	^{14}C-LPS bound (% total)[b]	Precipitate with LPS[c]
Wheat germ agglutinin	8.7	9.0	−
Soybean agglutinin	6.1	21.1	−
Potato agglutinin	>11.0	95.0	−
Polylysine	>11.0	61.2	+
Histone (corn)	>11.0	85.0	+
Lysozyme (egg white)	11.35	4.2	−
Bovine serum albumin	4.7	25.0	−

[a] 10 μg per sample.
[b] Average of three samples.
[c] 200 μg of protein and 200 μg of LPS per well.

lysozyme, however, is puzzling in light of its extremely high pI (11.35). It is possible that the distribution of charges on the protein, as well as the overall net charge, is important in the binding of LPS.

Several ionizable groups on LPS and EPS might be involved in binding. Phosphate and 2-keto-3-deoxyoctulosonic acid are present in *P. solanacearum* LPS and are known to contribute significantly to LPS charge (35). However, there are no detectable amounts of hexuronic acids, pyruvate, or phosphate in EPS (data not included), all of which are known to contribute to the acidity of bacterial heteropolysaccharides (36). As suggested by the weak interaction of EPS with the agglutinin, the density of charged groups may be low, making their detection difficult by ordinary methods.

Other agglutinins of phytopathogenic bacteria have been described, and for which no simple saccharide hapten has been found (6, 10, 11, 18, 23, 29). Romeiro et al. (23) concluded that ionic interactions could explain the agglutination of avirulent *Erwinia amylovora* by an agglutinin from apple xylem. Although our results suggest that electrostatic interactions are involved in potato agglutinin-LPS and -EPS interactions, the possibility that sugar-specific binding also contributes to the interaction cannot be ruled out. There is evidence that ionized groups can influence lectin affinity for a receptor (19, 31). When lectins bind to a complex molecule, charged groups adjacent to the site of lectin binding can influence avidity if the lectin possesses charged groups that can interact. The galactose-binding lectins of *Dictyostelium discoideum* can bind either to specific saccharide receptors or to ionic receptors on the surface of *Dictyostelium* spp.; the latter interaction can be distinguished by its reversal at high ionic strength (2).

Our experiments in vitro indicate that ionic conditions may be critical for binding of EPS to the agglutinin. It is possible that the ionic strength of intercellular fluid in vivo is too high for efficient binding of EPS to agglutinin. Estimates based on fluid recovered from water-infiltrated tobacco leaves indicate that undiluted intercellular fluid may be as high as 0.1 M ionic strength (P. Kraus, unpublished data). Little, however, is known about the precise composition of intercellular fluid.

Although the agglutinin may not be a lectin, it still may play a role in the observed attachment of avirulent bacteria to tobacco tissues, as it is found at sites on the cell wall in which it could interact with bacteria (15). The interaction of *P. solanacearum* with tobacco leaf cell walls in vitro is also inhibited at high ionic strength (9). It is difficult to envision how this binding interaction on the cell wall surface could trigger a host recognition response, however. It is more likely that the specificity of the interaction results from events occurring after attachment.

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