AN ABSTRACT OF THE THESIS OF

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Title: Characterization of a Bacterial Agglutinin from Legumes

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Leaves of Phaseolus vulgaris cv. 'Dark Red Kidney' and cv. 'Red Mexican U. I. 31', Phaseolus lunatus cv. 'Fordhook Bush 242', Phaseolus mungo cv. 'Berken', and Glycine max cv. 'Wilkin' have been shown to contain a factor which consistently agglutinated the saprophyte Pseudomonas putida; another saprophyte Pseudomonas fluorescens was agglutinated variably. The agglutinin was not active against phytopathogenic species of Pseudomonas, Agrobacterium, Erwinia, Corynebacterium, and Xanthomonas, and the saprophytic species Escherichia coli. Agglutinin from Dark Red Kidney and Red Mexican did not distinguish between races of Pseudomonas phaseolicola. The agglutinin may be specific for saprophytic Pseudomonads and responsible for their attachment and immobilization at the plant cell wall. More saprophytic Pseudomonads need to be tested.

Isolation and purification of agglutinin involved homogenization of leaves in water, centrifugation, ion exchange chromatography, ethanol precipitation, dialysis and either gel filtration on Sepharose® 6B-100 alone or an affinity purification using P. putida followed by gel filtration. Exclusion of agglutinin activity in the void volume indicated that the agglutinin has a molecular weight of a least 4×10^6

daltons. Results of colorimetric assays of active column fractions indicated that the agglutinin is a glycoprotein composed of protein, hexose and uronic acid. From gas chromatographic analyses of alditol-acetate derivatives of agglutinin arabinose and galactose were identified as the major neutral sugars present in the carbohydrate component. Sensitivity of the agglutinin to periodate indicated that the carbohydrate component of the agglutinin is essential for its activity. Agglutinin is active against only live bacteria and requires the divalent cation Mg^{++}. In the purest agglutinin preparations obtained less than one ug each of protein, hexose, and uronic acid was required to agglutinate 10^8 cells of P. putida.

Characterization of a Bacterial Agglutinin from Legumes

by

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CHARACTERIZATION OF A BACTERIAL AGGLUTININ FROM LEGUMES

I. INTRODUCTION

Bacteria enter plants through natural openings and wounds, but not all which enter cause disease. The plant defends itself against all but pathogens, parasites which initiate disease symptoms in the host.

The specificity of a host-parasite interaction is determined by the genetic constitutions of both host and parasite. Resistance and susceptibility are plant responses to a particular isolate of a parasite. Similarly avirulence and virulence are properties of the parasite which determine its ability to establish itself in a host plant. The interaction between a virulent parasite and a susceptible host results in the development of symptoms and disease. The interaction between an avirulent isolate and a resistant host results in little to no symptom development, symptoms often being the result of a hypersensitive response by the host (19).

The hypersensitive response is a rapid reaction of a host plant to an invading avirulent parasite. Saprophytic bacteria do not initiate this response. Macroscopically hypersensitivity is manifested as anything from necrotic flecking to confluent necrosis. Examination with the light microscope identifies this macroscopic necrosis as the cumulative product of single host cell responses. Avirulent bacteria inducing the response do not enter the host cell, but remain in the intercellular spaces (58). In responding host cells the cytoplasm becomes granulated and browning occurs due to the accumulation of polyphenols (39); these cells die. At the resolution of the electron

microscope, cells undergoing the hypersensitive response exhibit marked disruption of the plasmalemma and other membranous, subcellular organelles, eg. mitochondria and chloroplasts (24,39). The occurrence of electrolyte leakage (23,39) is also indicative of membrane damage. Penetration of a host cell by the hypha of an avirulent fungal isolate also initiates a hypersensitive response (43,54) which is similar to that initiated by an avirulent bacterium.

A. Encapsulation Phenomenon

Although hypersensitivity may account for the resistance of some hosts to avirulent bacteria (39), this has not been rigorously proven. Hypersensitivity also cannot account for the presence of bacteria latent within the plant such as saprophytes.

Another possible defense mechanism is the encapsulation of avirulent and saprophytic bacteria at the plant cell wall. This theory is suggested by several electron microscopy studies (16,25,27,28,46,50). It appears that an avirulent or saprophytic bacterium within the intercellular spaces of a leaf becomes attached to the plant cell wall. Disruption of the plant cell wall occurs and fibrillar material, presumably of plant cell wall origin, begins to surround the bacterium. An avirulent or saprophytic bacterium becomes completely engulfed in a fibrillar and granular matrix within six to nine hours after infiltration. As encapsulation progresses the plasmalemma of the responding plant cell(s) is disrupted and membrane vesicles accumulate near the site of plant cell wall activity. The process effectively immobilizes the bacterium and curtails its multiplication and spread.

Table I on page 4 summarizes the results of electron microscopy studies conducted on apple petioles, tobacco callus and leaves of tobacco, cotton, and bean, which had been infiltrated with various pathogenic, avirulent, and saprophytic bacterial isolates.

The first report of encapsulation of bacteria by a host was that of Huang, Huang, and Goodman (27). An avirulent strain of Erwinia amylovora was totally immobilized within the xylem vessel elements of apple petioles by granular vesicular material, possibly an agglutinating substance secreted by the ray parenchyma into the vessels. A virulent strain was not affected and continued to spread into the petiole and caused a susceptible response.

In tobacco leaves Goodman, Huang, and White (25) observed that the pea pathogen Pseudomonas pisi, which is nonpathogenic in tobacco, and the saprophyte Pseudomonas fluorescens were encapsulated at the mesophyll cell walls in the intercellular spaces, while the tobacco pathogen Pseudomonas tabaci remained free and multiplied extensively. Similarly on tobacco callus (28) P. pisi and P. fluorescens became localized in a meshwork of fibrillar material from the callus cell walls. Both isolates multiplied at a low level, that of the saprophyte being less than that of avirulent P. pisi. The virulent pathogen P. tabaci completely overran the callus. Sequeira, Gaard and DeZoeten (50) have found comparable results in tobacco leaves with a different array of bacterial isolates. An avirulent isolate of Pseudomonas solanacearum and two saprophytes, Bacillus subtilis and Escherichia coli are encapsulated, whereas a virulent isolate of P. solanacearum is not. Multiplication of avirulent and saprophytic isolates is limited, whereas that of virulent isolates is extensive.

TABLE I. ENCAPSULATION OF BACTERIA BY PLANT CELL WALLS

PLANT SP.	ORGAN/TISSUE	BACTERIA SP.	ENCAPSULATION	TISSUE REACTION	REFERENCE
Malus sylvestris cv. 'Jonathan'	xylem/petiole	Erwinia amylovora str. E8	+	HR	(27)
		str. E9	-	S	
Nicotiana tabacum cv. 'Samsun NN'	leaf	Pseudomonas tabaci	-	S	(25)
		Pseudomonas pisi	+	HR	
		Pseudomonas fluorescens	+	None	
cv. 'Bottom Special'	leaf	Pseudomonas solanacearum			(50)
		K60-B1	+	HR	
		K60	-	S	
		Bacillus subtilis	+	None	
		Escherichia coli	+	None	
cv. 'Samsun NN'	callus	Pseudomonas tabaci	-		(28)
		Pseudomonas pisi	+		
		Pseudomonas fluorescens	+		
Gossypium hirsutum cv. 'Im 216'	cotyledon	Xanthomonas malvacearum (Race 1)	+	HR	(16)
cv. 'Ac 44'			-	S	
Phaseolus vulgaris cv. 'Red Mexican'	leaf	Pseudomonas phaseolicola (Race 1)	+	HR	(46)
cv. 'Canadian Wonder'			-	S	

+ = ENCAPSULATION - = NO ENCAPSULATION HR = HYPERSENSITIVE RESPONSE S = SUSCEPTIBLE REACTION

Studies in cotton and bean have investigated the reaction of a race of a pathogen on differential cultivars. In the cotton cultivar Im 216, which is resistant to Race 1 of Xanthomonas malvacearum, a Race 1 isolate of this pathogen becomes completely engulfed by fibrillar and granular material in the intercellular spaces. This same Race 1 isolate in the susceptible cultivar Ac 44 is not encapsulated (16). Likewise, a Race 1 isolate of Pseudomonas phaseolicola becomes partially encapsulated by the cell walls of leaf in the intercellular spaces of the resistant bean cultivar Red Mexican, but remains free in the intercellular spaces of the susceptible bean cultivar Canadian Wonder (46).

The degree to which encapsulation affects the viability of entrapped bacteria is uncertain. An avirulent strain of E. amylovora appears to be degraded after immobilization within the xylem vessel elements of apple petioles (27). The saprophyte P. fluorescens when enmeshed by tobacco callus cell walls is also damaged. Spherical bodies, one per trapped bacterium, appear to evaginate from the bacterial cell wall and lyse (28). These two observations suggest that encapsulation, or more likely a succeeding active plant response, is detrimental to avirulent and saprophytic bacteria. Perhaps enzymes secreted locally by host cells are responsible for the lysis of P. fluorescens and degradation of avirulent E. amylovora.

Phytoalexins may have a further antibacterial effect on encapsulated avirulent bacteria. Both legumes and solanaceous plants are known to produce phytoalexins in response to infection by avirulent isolates of fungi (5,8,13,44,17,30,59) and viruses (4,6,9,7,14). Gnanamanickan and Patil (21,22) have demonstrated the production of the isoflavanoids

phaseollin, phaseollidin, phaseollinisoflavan, kievitone, and coumesterol in resistant bean cultivars inoculated with avirulent isolates of P. phaseolicola. Colony growth of isolates of P. phaseolicola and Pseudomonas glycinea were inhibited to various degrees by these compounds in vitro (21,38). The saprophyte P. fluorescens was relatively insensitive to these phytoalexins. As of yet direct proof of antibacterial activity of phytoalexins in vivo has not been obtained.

Encapsulation may be a method of keeping saprophytes at a tolerable level within the host. In bean inoculated with P. fluorescens the viable population level remained fairly constant for at least seven days after inoculation (39). A slight increase in population of P. fluorescens is seen in tobacco leaves and callus (28).

The relation of the encapsulation phenomenon to the hypersensitive response is also not fully understood. Avirulent isolates become encapsulated and initiate a hypersensitive response. Heat-killed avirulent isolates and saprophytes are also encapsulated (50), but do not induce a hypersensitive response (50,39). This would indicate that encapsulation alone does not trigger the hypersensitive response, since a live avirulent bacterium is requisite for the response by the plant cell. Stall and Cook (56) have indirect evidence that bacterial-plant cell contact is necessary for a hypersensitive response to occur. Possibly contact facilitates the passage of some substance from the live avirulent bacterium to the host cell which elicits hypersensitivity. The hypersensitive response is exemplified by disruption of the plasmalemma with vesiculation followed by extensive disintegration of cell organelles (24). Plant cells to which avirulent bacteria have become encapsulated incur this degree of membrane damage. Although some plasma-

lemma disruption and vesiculation take place with encapsulation of saprophytes, further cell organelle disorganization does not occur. Vesicle accumulation at the plasmalemma near the site of encapsulation probably denotes transport of material required for the process.

B. Plant and Bacterial Recognition Factors

The theory of host-parasite recognition proposed by Albersheim and Anderson-Prouty (2) is essentially the expression and interaction of pathogen avirulence and host resistance genes at the molecular level. Products of a plant's resistance genes, possibly receptor proteins in the plasmalemma, recognize carbohydrate "antigens" on the surface of the pathogen, these "antigens" being the products of the avirulence genes of a pathogen.

The encapsulation phenomenon is probably dictated by a similar recognition event. The attachment of avirulent and saprophytic bacteria to the plant cell wall could be precipitated in at least two ways.

1) A receptor in the plant cell wall directly interacts with an "antigen" on the cell wall of the bacterium.

or 2) There is a free component which possesses a binding site for a bacterial cell wall "antigen" and a plant cell wall "antigen". This "bridge" component could be of bacterial or plant origin. If of plant origin such a "bridge" component may have different binding sites which recognize different bacteria.

Which of these two situations actually exists is unknown. Present evidence would support either model, although no one has yet demonstrated the existence of a free "bridge" component. Possibly such a component is being secreted by the vesicles which accumulate adjacent

to a site where encapsulation is in progress.

Lectins are proteins, glycoproteins or lipoproteins which recognize and bind specific glycosyl moieties (52,36). Most of the presently known lectins have been obtained from plants (36). The role of lectins in recognition and host-parasite specificity has been discussed in two recent review articles (15,49).

Lectins have been implicated as the plant recognition factor in the encapsulation phenomenon. Potato lectin purified from tubers of Solanum tuberosum cv. 'Katahdin' was bioassayed for agglutination activity against a collection of P. solanacearum isolates representative of all three races and a diverse host range. All 34 avirulent isolates tested were agglutinated by potato lectin, whereas the 55 virulent isolates tested were not (51). These results correlated with those of the companion electron microscopy study in tobacco leaves (50) in which an avirulent isolate of P. solanacearum was completely encapsulated and a virulent isolate was not.

If potato lectin is responsible for the attachment of avirulent bacteria to the cell wall in solanaceous plants, it should be present at the plant cell wall. The presence of potato lectin at this location has been demonstrated in leaves and stems of potato by fluorescent antibody techniques (33).

At present the plant recognition factor functioning in encapsulation has not been isolated and purified from leaves, the organ in which most of the electron microscopy studies have been conducted. Since encapsulation occurs on tobacco callus, the plant recognition factor may be common to parenchyma cell walls present in all organs.

The "antigen" on the bacterial cell wall which interacts with the host cell wall receptor is believed to be a lipopolysaccharide component (26,40,41). Purified lipopolysaccharide of avirulent P. solanacearum binds to mesophyll cells of tobacco when infiltrated into the intercellular spaces of leaves; the plasmalemma and plant cell wall undergo the same changes as when a heat-killed bacterium binds to the plant cell wall (50,26).

Virulent isolates of P. solanacearum also have this lipopolysaccharide component present on their cell walls. However, virulent strains secrete great quantities of extracellular polysaccharide which effectively masks the lipopolysaccharide "antigen" so that the plant cell wall receptor cannot recognize it (51).

The encapsulation of saprophytic and avirulent bacteria is a probable defense mechanism in plants. The phenomenon involves three components: 1) a plant cell surface receptor, possibly a lectin, 2) the extracellular polysaccharide slime layer of certain virulent bacteria which prevents recognition and encapsulation, and 3) the lipopolysaccharide component on the bacterial cell wall which may elicit encapsulation, provided it is available for binding by the plant lectin. Although lipopolysaccharide may elicit encapsulation, which effectively prevents multiplication of the bacteria within the host, lipopolysaccharide does not elicit the hypersensitive response itself. Some other factor from live avirulent bacteria is required.

C. Purpose of the Study

Encapsulation of avirulent bacteria has been demonstrated in Phaseolus vulgaris (46), and bean leaves have been shown to contain factors which agglutinate bacteria (3). The purpose of this study was

to isolate and characterize an agglutinin from Dark Red Kidney and to see if a similar agglutinin could be found in other P. vulgaris cultivars, Phaseolus species and legumes. The degree of specificity of the agglutinin with respect to pathogenicity was also assessed by assaying a range of phytopathogenic and saprophytic bacterial isolates.

II. MATERIALS AND METHODS

A. Purification of Agglutinin

1. Plant Growth Conditions

Seed of cultivars of Phaseolus vulgaris, Phaseolus lunatus and Phaseolus mungo was obtained from the sources listed in Table II on page 12. Seed was rinsed in tapwater and soaked for five minutes in a 20% (v/v) solution of commercial bleach. After a tapwater rinse the seed was hand-sorted to remove culls. After another five minute soak in 20% (v/v) bleach, the seeds were directly planted in vermiculite at a 1 cm depth in plastic flats (52.5 cm x 26.0 cm x 6.5 cm) which had been surface-sterilized with 95% ethanol.

The flats were removed to a growth chamber set at 21°C and a 14 hour light/10 hour dark cycle. Germination and growth to the monofoliate leaf stage occurred in about two weeks for Dark Red Kidney and Red Mexican, whereas Fordhook Bush 242 and Berken required about one month to reach this stage. Plants were maintained with water and nutrient solution. Leaves were harvested at the monofoliate stage.

Leaves from the soybean cultivar Wilkin, grown in the greenhouse, were kindly donated by Dr. Harold Evans, Laboratory for Nitrogen Fixation Research, Oregon State University. Trifoliate leaves from plants which had reached the flowering stage were used in this study.

TABLE II. SEED SOURCES

Bean	Source
Phaseolus vulgaris Dark Red Kidney Red Mexican U. I. 31	Idaho Seed Company P. O. Box 1072 Twin Falls, Idaho 83301
Phaseolus lunatus Fordhook Bush 242	Stokes Seeds Inc. 737 Main St. P. O. Box 548 Buffalo, NY 14240
Phaseolus mungo Berken	W. Atlee Burpee Co. Riverside, CA 92502

2. Culture of Bacteria

Stock cultures of the bacteria used in this study (Table III, page 13) were maintained on plates of rich media at room temperature and were transferred at monthly intervals. Bacteria used in agglutinin bioassays and the affinity procedure were grown in liquid rich medium on a gyratory shaker at room termperature.

Rich media contained 10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 0.5 g sodium citrate·$2H_2O$, 1.0 g $(NH_4)_2SO_4$, 10.0 g nutrient broth (Difco Bacto, Difco Laboratories, Detroit, Michigan), 6.0 g yeast extract (Difco Bacto) per liter distilled water. Fifteen grams of agar (Type IV, Sigma Chemical Co., P. O. Box 14508, St. Louis, MO 63178), per liter, was added for plates. The mixture was autoclaved for 20 minutes at 121°C. Just prior to use 10 ml sterile 20% (w/v) sucrose and 1 ml sterile 1M $MgSO_4$ were added per liter liquid medium.

Bacteria were grown in 500 ml culture flasks, each containing 100 ml

TABLE III. SOURCES OF BACTERIAL ISOLATES

Bacterial Isolate	Source+
Agrobacterium radiobacter 84	(a)
Agrobacterium rhizogenes A4	(a)
Corynebacterium sepedonicum	(b)
Erwinia carotovora var. atroseptica SR 6	(b)
Erwinia carotovora var. atroseptica SR 31	(b)
Erwinia carotovora var. carotovora SR 24	(b)
Erwinia carotovora var. carotovora SR 189	(b)
Escherichia coli CSH 125 Sup F thi$^-$	(c)
Pseudomonas fluorescens	(a)
Pseudomonas glycinea 100	(c)
Pseudomonas lachrymans	(a)
Pseudomonas marginalis	(a)
Pseudomonas morsprunorum	(a)
Pseudomonas phaseolicola	
G50	(d)
G51	(d)
HB20	(d)
HB28	(d)
HB51	(d)
PP	(e)
Pseudomonas putida	(a)
Pseudomonas solanacearum	(f)
Pseudomonas syringae	(a)
Pseudomonas tabaci	(a)

+Sources:

(a) Dr. L. Moore, Dept. of Botany and Plant Pathology, Oregon State University

(b) Dr. M. Powelson, Dept. of Botany and Plant Pathology, Oregon State University

(c) M. Curiale, Dept. of Botany and Plant Pathology, Oregon State University

(d) Dr. N. Pananapolous, Dept. of Plant Pathology, University of California-Berkeley

(e) Dr. A. J. Anderson, Dept. of Botany and Plant Pathology, Oregon State University

(f) J. McMorran, Dept. of Botany and Plant Pathology, Oregon State University

of liquid rich media. Each flask was inoculated with one standard loop-ful of bacteria scraped from a stock culture, and placed on a gyratory shaker at room temperature. Cells were harvested at a given time after inoculation (usually at 15 hours) by centrifugation at 12,100 x g for 10 minutes at 4°C. The bacterial pellet was washed twice by suspension in sterile distilled water (SDW) and recentrifugation. Bacterial pellets prepared in this manner were used in the affinity purification procedure. For use in the agglutinin bioassay the bacterial pellet was suspended in 10 ml of SDW which yielded a suspension of about 10^{10} cells /ml as determined by dilution plating.

3. Extraction

Monofoliate leaves of bean were harvested and weighed. Leaves were homogenized in 100 g aliquots with SDW (1:1 (w/v), g leaves:ml SDW) using a Waring blender. The homogenate was filtered through four layers of cheesecloth, and the resulting filtrate was centrifuged at 12,100 x g for 10 minutes at 4°C. This centrifugation precipitated any cell wall material that had passed through the cheesecloth and other cellular debris. The pellet was discarded and the supernatant was used as the crude extract.

4. Ion Exchange Chromatography

The crude extract was subjected to batch ion exchange resin treatments. DEAE Sephadex® A-50-120 and CM Sephadex® C-50-120 (Sigma Chemical Co.) were prepared by hydration in distilled water followed by washes of 1M NaCl, 95% ethanol, and distilled water in a sintered glass funnel. The crude bean extract was first mixed with DEAE Sephadex resin (3:1 (v/v), Crude:resin) and placed on a gyratory shaker for 20 minutes

at room temperature. The DEAE filtrate, obtained by vacuum filtration, was mixed with CM Sephadex resin (3:1 (v/v), DEAE filtrate:resin) and the slurry was shaken for 20 minutes at room temperature.

5. Ethanol Precipitation

The CM filtrate, obtained by vacuum filtration of the slurry, was mixed with pre-chilled (4°C) 95% ethanol (1:3 (v/v), CM filtrate:95% ethanol). The precipitate, formed by standing at 4°C or -6°C overnight, was retrieved by centrifugation at 14,700 x g for 10 minutes at 4°C.

The ethanol precipitate was processed by one of two methods:

1) Pellets were resuspended in 100 to 200 ml distilled water and dialyzed against cold running tapwater for 12 to 24 hours. Undissolved material was removed by centrifugation at 12,100 x g for 10 minutes at 4°C. The supernatant was saved.

2) Pellets were resuspended in 100 ml distilled water at room temperature in a Waring Blender for 30 seconds to one minute. A few drops of octanol were added and the mixture was blended one minute. The suspension was centrifuged at 12,100 x g for 10 minutes at 4°C, and the supernatant was dialyzed against cold running tapwater for 12 to 24 hours. The pellet, obtained by centrifugation of the octanol treated suspension, was also resuspended in 100 ml distilled water and dialyzed against cold running tapwater for 12 to 24 hours. Each of these dialysates was clarified by centrifugation at 12,100 x g for 10 minutes at 4°C.

The clarified dialysate was further purified by either: 1) gel filtration on Sepharose® 6B-100 (Sigma Chemical Co.) or 2) affinity purification utilizing P. putida followed by gel filtration on Sepharose® 6B-100.

6. Gel Filtration

Fifty milliliters of dialysate was concentrated to two to five milliliters by rotary evaporation at 30 to 35°C. This sample was applied to a column of Sepharose® 6B-100 (23.0 cm x 2.1 cm) which had been pre-equilibrated with 1mM $MgCl_2$. The column was eluted with 1mM $MgCl_2$ at room temperature and fractions of 80 drops each were collected.

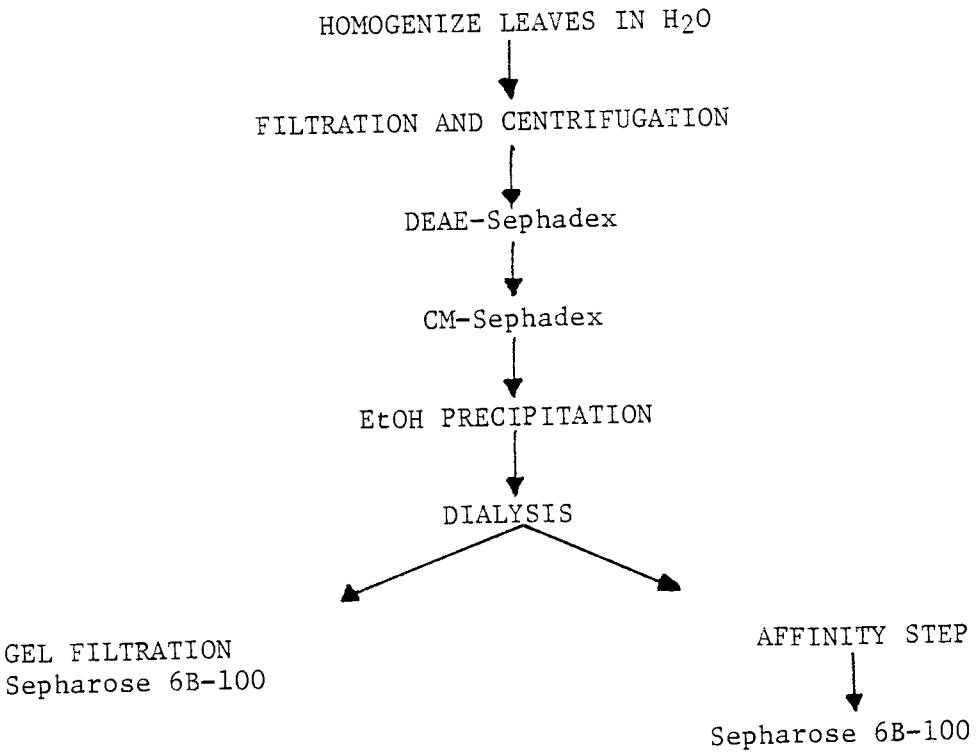

Figure 1. Purification of agglutinin from bean or soybean leaves.

7. Affinity Purification Procedure

The affinity procedure utilized P. putida to extract the agglutinin. One hundred to 150 ml of dialysate was adjusted to 1mM Mg^{++} by the addition of 0.1M $MgCl_2$ and mixed with two cultures (approximately 4×10^{11} cells) of P. putida. (For growth and harvest conditions see 'Culture of Bacteria' on pages 12 and 14.) After mixing on a gyratory shaker for 15 minutes at room temperature, the suspension was centrifuged at 12,100 x g for 10 minutes at 4°C. The bacterial pellet was saved and the supernatant was treated by two more affinity cycles, with the addition of $MgCl_2$ and two fresh bacterial pellets at the beginning of each cycle. The bacterial pellets were washed with SDW to release the agglutinin into solution. This process was achieved by suspending the pellets in 100 ml SDW, allowing a 15 minute incubation on a gyratory shaker, and centrifugation at 12,100 x g for 10 minutes at 4°C. The process was repeated two to three times. All of the washes were pooled and filter sterilized by passage through a membrane with a pore size of 0.4 or 0.45 μ (Millipore 0.45 μ or Bio-Rad Unipore 0.4 μ). Concurrent with manipulation of the bean or soybean dialysate sample, a control of SDW adjusted to 1mM Mg^{++} was similarly processed to determine any contribution of the bacteria to the final extracts. Two hundred milliliters of cell wash from bean or soybean dialysate and the control wash were concentrated to two milliliters by rotary evaporation at 30 to 35°C and chromatographed on Sepharose® 6B-100 using previously described conditions (see 'Gel Filtration' on page 16).

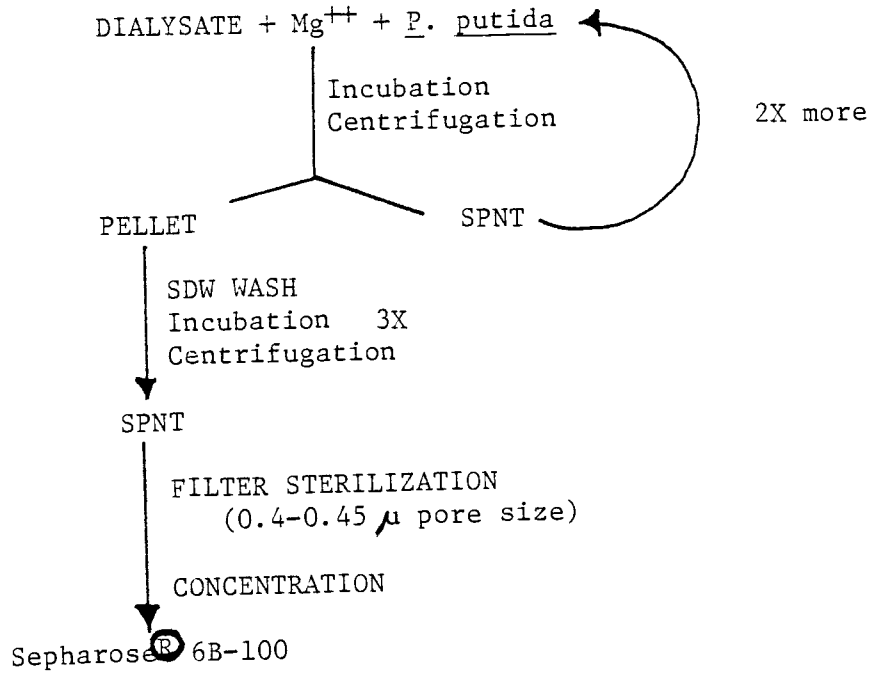

Figure 2. Purification of agglutinin by Pseudomonas putida affinity procedure.

B. Characterization of Agglutinin

At each stage in the purification the preparation was assayed for units of agglutinin activity against P. putida and concentration of protein, hexose, and uronic acid as determined by colorimetric assays. Samples of the clarified dialysate of the ethanol precipitate, the extracted supernatant and bacterial cell washes (bean and control) of the affinity procedure, and pooled active column fractions were dried down in a filtered air stream for acetylation to procure additional information about the carbohydrate component of the agglutinin. Details of these procedures follow.

1. Agglutinin Bioassays

Agglutinin activity was assayed by measuring the ability of preparations to cause cells of P. putida to clump together. For determination of units of agglutinin activity through the steps of purification, P. putida cells grown in rich media shake culture and harvested at 15 hours were used. Extracts were bioassayed by a tube agglutination test at the following dilutions: undiluted, 1/2, 1/10, 1/20, 1/100, 1/200, 1/1000, 1/2000, and 1/10,000. Units of agglutinin activity are defined as the reciprocal of the dilution of the most dilute solution which caused agglutination of P. putida. Each assay tube (diSPo^{TM}, 13 x 100 mm glass culture tubes, Scientific Products) contained 500 µl test solution, 150 µl 1mM $MgCl_2$, and 50 µl bacterial suspension (approximately 10^{10} cells / ml SDW). Racks of tubes were placed on a gyratory shaker for 15 minutes at room temperature, after which time they were read visually for the appearance of a fine, white, grainy precipitate, the agglutinated bacteria. An opaque white suspension indicated a lack of agglutination. Control tubes containing 500 µl SDW, 150 µl 1mM $MgCl_2$, and 50 µl bacterial suspension were run simultaneously.

Agglutinin preparations were also assayed with heat-killed P. putida cells to determine if the agglutination process required live cells. A SDW suspension of 15 hour P. putida cells was autoclaved at 121°C for 20 minutes to kill the cells. After cooling to room temperature, this suspension of dead P. putida was used following bioassay conditions described above.

To determine the specificity of the agglutinin with respect to pathogenicity of bacteria, all of the bacterial isolates listed in

Table III on page 13 were assayed with partially purified agglutinin from Dark Red Kidney, Red Mexican, and Wilkin. Bacteria were grown in rich media liquid shake culture and harvested at 15 hours. Corynebacterium sepedonicum was harvested at 30 hours since it multiplied more slowly than the other isolates. The agglutinin preparations, the dialysate of the ethanol precipitate step, were assayed at full strength and a 1/10 dilution. Assay conditions were as previously described on page 18.

2. Colorimetric Assays

Protein, hexose, and uronic acid in agglutinin preparations were determined by colorimetric assays. Protein concentration was measured by the procedure of Lowry et al. (37) with the sample incubation modification of McDonald and Chen (42) using bovine serum albumin (Sigma Chemical Co.) as the standard.

Hexose concentration was determined by using an anthrone assay adapted from Dische (20) and Spiro (55). Five hundred microliters of sample containing 10 to 100 μg/ml hexose was pipetted into a glass test tube at room temperature. One milliliter of anthrone reagent (0.2% (w/v) anthrone in concentrated H_2SO_4) was added and mixed rapidly. After heating in a 100°C water bath for five minutes, the samples were allowed to cool to room temperature before reading at 620 nm. Glucose (Sigma Chemical Co.) was used as the standard.

Uronic acid concentration was measured by using a carbazole assay modified from Bitter and Muir (10) and Dische (20). Two hundred microliters of sample containing 10 to 50 μg/ml uronic acid was pipetted

into a glass test tube at room temperature. One milliliter of 0.025M sodium tetraborate·10 H_2O in concentrated H_2SO_4 was added per tube. Samples were heated in a 100°C water bath for 25 minutes. After cooling the samples to room temperature, 50 µl of carbazole reagent (0.1% (w/v) carbazole in ethanol) were added per tube. Samples were heated in a 100°C water bath for 35 minutes. After cooling to room temperature they were read at 530 nm. α-D Galacturonic acid (Sigma Chemical Co.) was used as the standard.

For each of these assays the Zeiss PM2 DL Spectrophotometer was used to read the absorbance of the standard at the appropriate wavelength and to directly read concentration in µg/ml of protein, hexose, or uronic acid of each of the samples.

3. Alditol-acetate derivatization and identification

Neutral sugar composition of agglutinin preparations was determined by identification of their respective alditol-acetate derivatives. The procedure used was adapted from that of Jones and Albersheim (29).

Authentic samples of the following sugars composed the standard: 500 µg each of glucose, galactose, mannose, xylose, arabinose, ribose, fucose, rhamnose, and inositol. Agglutinin samples containing from 200 to 500 µg hexose as determined by the anthrone assay (see page 20) plus 500 µg of the internal standard inositol were dried down in new, clean, screwtop test tubes in a filtered air stream at 50°C, and 500 µl 2N trifluoroacetic acid was added to each. The tubes were tightly closed with teflon-lined caps and autoclaved for one hour at 121°C to achieve hydrolysis of the sugar linkages. Hydrolysates were evaporated in an air stream at 50°C. The aldoses were converted to alditols by reduction

with $NaBH_4$; 250 µl of freshly prepared 10 mg/ml $NaBH_4$ in 1M NH_4OH was added per tube, and the samples were allowed to sit at room temperature for one hour. The reaction was stopped by dropwise addition of glacial acetic acid which decomposes excess $NaBH_4$. Effervescence denotes complete reduction and excess $NaBH_4$. Samples were washed six times with approximately 500 µl aliquots of 10% (v/v) glacial acetic acid in methanol to remove boric acid; after each addition the sample was evaporated in a filtered air stream at 50°C. The acetic acid/methanol washes were followed with five 500 µl methanol washes.

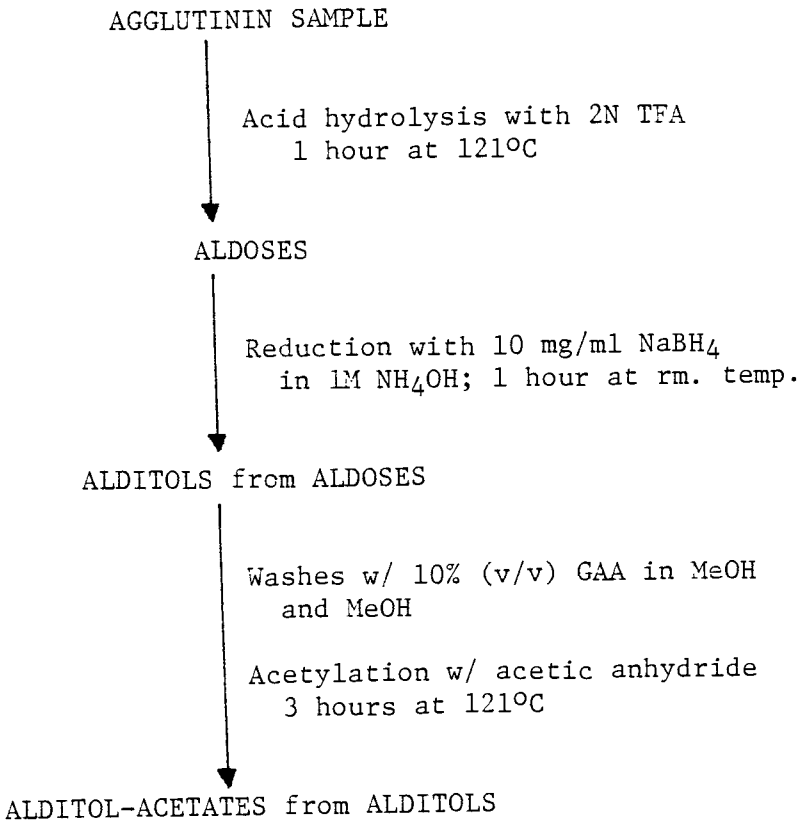

Figure 3. Derivitization scheme for GC analysis of aldoses in carbohydrate component of agglutinin.

Acetylation of the alditols to alditol-acetates was achieved by adding 250 µl acetic anhydride to each sample, tightly capping each tube, and autoclaving for three hours at 121°C. After autoclaving the alditol-acetates were extracted with chloroform. One milliliter of distilled water was added to each tube to hydrolyze the acetic anhydride to acetic acid. Two milliliters of chloroform was added; the chloroform layer was transferred to a new tube with a pasteur pipette. The water phase was extracted with an additional one milliliter of chloroform, this chloroform layer being pooled with the first. The chloroform extracts of the samples were evaporated in a filtered air stream at 50°C. Just prior to injection each sample residue was dissolved in 250 µl acetone (Reagent Grade) and three micorliters were injected into the gas chromatograph for analysis.

Acetylated samples were analyzed using Model 5830A Gas Chromatograph and Model 18850A GC Terminal, both products of Hewlett-Packard. The column material 3% SP-2340 on 100/120 Supelcoport 1-1863 (Supelco Inc., Supelco Park, Bellefonte, PA 16823) was packed by vibration and settling into copper tubing (I. D. = 0.32 cm, length = 1.83 m) plugged at both ends with glass wool. The column was conditioned for one hour prior to use with a N_2 flow rate of 50 ml/minute at 220°C. Injection conditions were: injection port 250°C, ionization detector 250°C, and a N_2 flow rate of 50 ml/minute. Run conditions were as follows. Upon injecting a sample the column was maintained at 175°C for five minutes, the temperature then increased at a rate of 2°/minute to 220°C and was maintained at 220°C for 20 minutes.

The Hewlett-Packard system used identified the neutral sugars present in the samples by identifying the retention times of their

alditol-acetate peaks relative to the retention time of the internal standard peak, inositol hexaacetate. The system integrated component peaks and calculated the amount of each neutral sugar in a sample as µg/sample tube. Each sugar has an area/amount ratio determined from its peak in the standard tube. This ratio is used to calculate µg of the sugar present in the sample and the area/amount ratio of the internal standard inositol equates the samples.

4. Periodate Treatment

Samples of agglutinin were treated with sodium meta-periodate to determine whether oxidations in the carbohydrate component of the agglutinin would alter its activity. Two milliliter samples of the ethanol precipitate dialysate and the affinity purified agglutinin were adjusted to 0.05M periodate by addition of solid sodium meta-periodate. The samples were shaken to dissolve the reagent and placed at 4°C overnight. Periodate treated samples were dialyzed against two liters of distilled water at 4°C with two changes of two liters each. Dialyzed samples and non-periodate treated samples were assayed against P. putida for agglutination activity under the conditions described on page 19.

5. Interaction of Bean Cultivars with Pseudomonas phaseolicola isolates

Two week old bean plants (see page 11 for germination and growth conditions) of Dark Red Kidney and Red Mexican U. I. 31 were inoculated with the isolates of P. phaseolicola listed in Table III on page 13 to determine host-pathogen interaction symptomatology for each isolate. Dark Red KIdney is susceptible to both races of P. phaseolicola, whereas Red Mexican U. I. 31 is resistant to Race 1 and susceptible to Race 2 isolates (45). The P. syringae isolate was also tested to assess its

pathogenicity in bean.

Bacteria grown in liquid rich media (see pages 12 and 14 for growth and harvest conditions) were harvested at 15 hours. Bacterial pellets from four 100 ml cultures were resuspended in SDW to a final concentration of approximately 2×10^9 cells/ml; total volume of inoculum was about 500 ml. Six plants of each cultivar were infiltrated per P. phaseolicola and P. syringae isolate. Controls were infiltrated with SDW. Infiltration was achieved as follows. A 600 ml beaker containing six bean plants submerged apex down in an inoculum suspension was placed inside a vacuum dessicator. This chamber was connected to a water aspiration system and a vacuum was applied for two five minute periods. The infiltrated plants were repotted in vermiculite, three plants per styrofoam cup. Potted plants were set in plastic trays, watered with nutrient solution, and the whole was enclosed in a large plastic bag to maintain a high humidity. Trays were transferred to the growth chamber and kept under the same conditions as for growing beans (see page 11). After 48 hours the plastic bags were removed. Symptoms developed in one to two weeks at which times symptoms were evaluated.

III. RESULTS

A. Purification

Extracts of homogenized legume leaves contained an agglutinin activity for Pseudomonas putida cells. Centrifugation of the crude homogenate and batch ion exchange chromatography with DEAE- and CM-Sephadex® increased the fold of purification of agglutinin activity with respect to protein and carbohydrate only slightly. (Refer to Tables IV through X.) DEAE-Sephadex® removed brown-colored materials possibly phenolics. The agglutinin did not bind to DEAE- or CM-Sephadex®. Total units of activity in the crude extract, DEAE filtrate, and CM filtrate were approximately the same. Therefore, units of activity applied to each resin were recovered 100% in the filtrate. One molar NaCl washes of the used resins contained no agglutinin activity; these salt washes had to be dialyzed prior to bioassay, because salt inhibited agglutination of P. putida by the plant factor.

Ethanol precipitation of the CM filtrate was used to remove chlorophylls. An active fraction was obtained, although recovery was low. Whether or not octanol was used to resuspend the pellets did not correlate with this loss. (Refer to Tables IV through X.) Loss of activity may be due to a number of causes. A very low percentage of precipitated agglutinin may have gone back into solution. Dialysis against water may have destroyed agglutinin activity; possibly dialysis against 1mM $MgCl_2$ would be a better choice, as agglutinin activity is enhanced by Mg^{++}. Possibly the activity values in the crude extract, DEAE filtrate, and CM filtrate are inflated. Despite the initial centrifugation step, these preparations contained particulate matter such

as plant cell membranes and wall fragments. Also if the agglutinin were bound to plant cell membranes or the cell wall, it may not be released but only precipitated and/or inactivated by the ethanol treatment. Sonication and solubilization of bean cell membranes and cell walls should be tried.

Gel filtration removed a considerable amount of inactive protein from the dialysate of the ethanol precipitate. Red Mexican and Fordhook Bush 242 showed the greatest fold purification of active protein, 15.0- and 23.0-fold respectively. Wilkin soybean and Dark Red Kidney were comparable at 9.9 and 8.4-fold purification of protein respectively. Gel filtration resulted in an agglutinin preparation with about half the amount of applied hexose and uronic acid. An additional loss in overall yield of activity after gel filtration may be due to the method by which samples were concentrated, ie. rotary evaporation at 30 to 35°C. Evaporation to dryness resulted in a loss of activity upon resuspension in distilled water. Excessive concentration of agglutinins may be causing the formation of high molecular weight aggregates with less activity. (See Tables IV, VI, VII, and IX.)

The affinity procedure exploited the Mg^{++} requirement of the agglutinin. Activity of a relatively pure agglutinin preparation was seemingly lost when treated with CM-Sephadex®; activity of the eluate was restored by addition of Mg^{++} to 1mM $MgCl_2$. Agglutinin was bound to live P. putida cells in the presence of Mg^{++} and was released when the Mg^{++} concentration was decreased by suspending agglutinated bacterial cells in sterile distilled water. Pseudomonas putida cells heat-killed by autoclaving at 121°C for 20 minutes are not agglutinated by the agglutinin.

Agglutinin was purified by the affinity procedure from preparations of Dark Red Kidney (Tables V and XI) and Wilkin (Tables X and XII). The affinity procedure coupled with gel filtration produced a Dark Red Kidney agglutinin sample with specific activities for protein and carbohydrate many times greater than those of Dark Red Kidney agglutinin purified by gel filtration alone. The active column fractions of affinity purified Dark Red Kidney agglutinin showed 14.4-, 4.6- and 2.7-fold purification for protein, hexose, and uronic acid respectively. The specific activities for agglutinin of Wilkin soybean purified by gel filtration alone or the affinity procedure plus gel filtration were comparable. The affinity procedure resulted in about a two-fold increase in specific acitvity with respect to hexose over that achieved by gel filtration alone. Overall yield of agglutinin by the affinity procedure coupled with gel filtration on Sepharose® 6B-100 was very poor, less than 1%. Poor yield may be due to inadequate extraction of agglutinin from dialysate, inefficient recovery of agglutinin from P. putida cells or loss of activity during filter sterilization of cell washes. All of the agglutinin activity present in the dialysate treated with P. putida and Mg^{++} was not extracted (Tables XI and XII). Determination of agglutinin activity demonstrated that all activity applied to the Sepharose® 6B-100 column was recovered.

TABLE IV. PURIFICATION OF AGGLUTININ FROM DARK RED KIDNEY

Prep	Volume (mls)	Corrected Volume (mls)	Units per 0.5mls	Total Units	Protein (ug/ml)	Total Protein (mg)	Hexose (ug/ml)	Total Hexose (mg)	Uronic Acid (ug/ml)	Total Ur. Ac. (mg)	Specific Activity: Units per mg Protein	Units per mg Hexose	Units per mg Ur. Ac.	Relative Purification: Protein	Hexose	Uronic Acid	Overall Yield of Activity %
Crude	335	335	200	$1.34x10^5$	25,600	8,580	3,000	1,005	1,150	385	16	133	348	1.0	1.0	1.0	100
DEAE	400	412	200	$1.65x10^5$	19,000	7,830	2,250	927	650	268	21	178	254	1.3	1.3	0.7	123
CM	420	444	200	$1.78x10^5$	17,000	7,550	1,800	799	700	311	24	222	571	1.5	1.7	1.6	133
Dialysate[a]	445	482	20	$1.93x10^4$	1,800	868	170	82	70	34	22	235	567	1.4	1.8	1.6	14
6B-100 Column																	
#4→10	106	254	20	$1.02x10^4$	300	76	98	25	53	13	134	406	782	8.4	3.1	2.2	8

a = Dialysate of ethanol precipitate.

TABLE V. PURIFICATION OF AGGLUTININ FROM DARK RED KIDNEY (AFFINITY PROCEDURE)

Prep	Volume (mls)	Corrected Volume (mls)	Units per 0.5mls	Total Units	Protein (ug/ml)	Total Protein (mg)	Hexose (ug/ml)	Total Hexose (mg)	Uronic Acid (ug/ml)	Total Ur. Ac. (mg)	Specific Activity: Units per mg Protein	Units per mg Hexose	Units per mg Ur. Ac.	Relative Purification: Protein	Hexose	Uronic Acid	Overall Yield of Activity %
Crude	570	570	2,000	$2.28x10^6$	23,600	13,500	1,800	1,026	880	502	169	2,220	4,540	1.0	1.0	1.0	100
DEAE	648	660	2,000	$2.64x10^6$	18,000	11,900	1,500	990	620	409	222	2,670	6.450	1.3	1.2	1.4	116
CM	696	720	2,000	$2.88x10^6$	17,200	12,400	1,150	828	540	389	232	3,480	7,403	1.4	1.6	1.6	126
Dialysate[a]	125	131	100	$2.62x10^4$	940	123	400	52	182	24	213	504	1,092	1.3	0.2	0.2	1
Affinity Step																	
Cell Wash[b]	383	672	1	$1.34x10^3$	3	2	4	3	1	0.7	670	447	1,910	4.0	0.2	0.4	0.06
6B-100Column																	
#6 → 8[b]	30	61	10	$1.22x10^3$	13	0.5	3	0.12	1	0.1	2,440	10,200	12,200	14.4	4.6	2.7	0.05

a = Dialysate of ethanol precipitate; octanol used in resuspension.
b = Bacterial control already subtracted.

TABLE VI. PURIFICATION OF AGGLUTININ FROM RED MEXICAN

Prep	Volume (mls)	Corrected Volume (mls)	Units per 0.5mls	Total Units	Protein (ug/ml)	Total Protein (mg)	Hexose (ug/ml)	Total Hexose (mg)	Uronic Acid (ug/ml)	Total Ur. Ac. (mg)	Specific Activity: Units per mg Protein	Units per mg Hexose	Units per mg Ur. Ac.	Relative Purification: Protein	Hexose	Uronic Acid	Overall Yield of Activity %
Crude	505	505	200	2.02×10^5	15,600	7,880	2,400	1,210	900	455	26	167	444	1.0	1.0	1.0	100
DEAE	575	587	200	2.35×10^5	11,200	6,570	1,600	939	500	294	36	250	799	1.4	1.5	1.8	116
CM	580	602	100	1.20×10^5	12,000	7,220	1,500	903	450	271	17	133	444	0.7	0.8	1.0	59
Dialysate[a]	302	319	20	1.28×10^4	2,650	845	180	57	90	29	15	224	440	0.6	1.3	1.0	6
6B-100 Column #7 → 10	85	136	10	2.72×10^3	51	7	67	9	32	4	389	302	680	15.0	1.8	1.5	1

a = Dialysate of ethanol precipitate.

TABLE VII. PURIFICATION OF AGGLUTININ FROM FORDHOOK BUSH 242 (LIMA BEAN)

Prep	Volume (mls)	Corrected Volume (mls)	Units per 0.5mls	Total Units	Protein (ug/ml)	Total Protein (mg)	Hexose (ug/ml)	Total Hexose (mg)	Uronic Acid (ug/ml)	Total Ur. Ac. (mg)	Specific Activity: Units per mg Protein	Units per mg Hexose	Units per mg Ur. Ac.	Relative Purification: Protein	Hexose	Uronic Acid	Overall Yield of Activity %
Crude	195	195	200	7.80×10^4	28,400	5,540	5,250	1,024	1,200	234	14	76	333	1.0	1.0	1.0	100
DEAE	210	228	200	9.12×10^4	19,000	4,330	3,150	718	840	192	21	127	475	1.5	1.7	1.4	117
CM	208	237	200	9.48×10^4	16,800	3,980	2,650	628	710	168	24	151	564	1.7	2.0	1.7	122
Dialysate[a]	210	251	10	5.02×10^3	740	186	122	31	50	13	27	162	386	1.9	2.1	1.2	6
6B-100 Column #7 → 10	38	95	10	1.90×10^3	59	6	50	5	27	3	316	380	633	23.0	5.0	1.9	2

a = Dialysate of ethanol precipitate.

TABLE VIII. PURIFICATION OF AGGLUTININ FROM BERKEN (MUNG BEAN)

Prep	Volume (mls)	Corrected Volume (mls)	Units per 0.5mls	Total Units	Protein (ug/ml)	Total Protein (mg)	Hexose (ug/ml)	Total Hexose (mg)	Uronic Acid (ug/ml)	Total Ur. Ac. (mg)	Specific Activity: Units per mg Protein	Units per mg Hexose	Units per mg Ur. Ac.	Relative Purification: Protein	Hexose	Uronic Acid	Overall Yield of Activity %
Crude	166	166	200	6.64×10^4	9,800	1,630	1,180	196	510	85	41	339	781	1.0	1.0	1.0	100
DEAE	180	186	200	7.44×10^4	6,800	1,270	840	156	320	60	59	477	1,240	1.4	1.4	1.6	112
CM	190	201	100	4.02×10^4	5,600	1,130	720	145	275	55	36	277	731	0.9	0.8	0.9	61
Dialysate[a]	128	139	10	2.78×10^3	392	54	114	16	55	8	51	174	348	1.2	0.5	0.4	4

a = Dialysate of ethanol precipitate.

TABLE IX. PURIFICATION OF AGGLUTININ WILKIN (SOYBEAN)

Prep	Volume (mls)	Corrected Volume (mls)	Units per 0.5mls	Total Units	Protein (ug/ml)	Total Protein (mg)	Hexose (ug/ml)	Total Hexose (mg)	Uronic Acid (ug/ml)	Total Ur. Ac. (mg)	Specific Activity: Units per mg Protein	Specific Activity: Units per mg Hexose	Specific Activity: Units per mg Ur. Ac.	Relative Purification: Protein	Relative Purification: Hexose	Relative Purification: Uronic Acid	Overall Yield of Activity %
Crude	1,500	1,500	2,000	6.00×10^6	18,800	28,200	4,050	6,080	1,067	1,600	213	987	6,080	1.0	1.0	1.0	100
DEAE	1,800	1,812	2,000	7.20×10^6	15,200	27,500	2,900	5,260	767	1,390	262	1,370	5,180	1.2	1.4	0.9	120
CM	1,980	2,004	2,000	8.00×10^6	12,000	24,000	2,450	4,910	567	1,140	333	1,630	7,020	1.6	1.7	1.2	133
Dialysate[a]	199	270	20	1.08×10^4	830	224	540	146	210	57	48	74	189	0.2	0.1	0.03	0.18
6B-100 Column #7 → 8(9)	23	71	20	2.84×10^3	15	1	36	3	14	1	2,840	947	2,840	13.3	1.0	0.5	0.05

a = Dialysate of ethanol precipitate; octanol used in resuspension.

TABLE X. PURIFICATION OF AGGLUTININ FROM WILKIN (SOYBEAN) (AFFINITY PROCEDURE)

Prep	Volume (mls)	Corrected Volume (mls)	Units per 0.5mls	Total Units	Protein (ug/ml)	Total Protein (mg)	Hexose (ug/ml)	Total Hexose (mg)	Uronic Acid (ug/ml)	Total Ur. Ac. (mg)	Specific Activity: Units per mg Protein	Specific Activity: Units per mg Hexose	Specific Activity: Units per mg Ur. Ac.	Relative Purification: Protein	Relative Purification: Hexose	Relative Purification: Uronic Acid	Overall Yield of Activity %
Crude	1,500	1,500	2,000	6.00×10^6	18,800	28,200	4,050	6,080	1,067	1,600	213	987	6,080	1.0	1.0	1.0	100
DEAE	1,800	1,812	2,000	7.20×10^6	15,200	27,500	2,900	5,260	767	1,390	262	1,370	5,180	1.2	1.4	0.9	120
CM	1,980	2,004	2,000	8.00×10^6	12,000	24,000	2,450	4,910	567	1,140	333	1,630	7,020	1.6	1.7	1.2	133
Dialysate[a]	83	333	20	1.33×10^6	670	223	390	130	200	67	60	102	199	0.3	0.1	0.03	22
Affinity Step Cell Wash[b]	285	1,144	1	2.29×10^3	7	8	4	4	1	1	286	572	2,290	1.3	0.6	0.4	0.04
6B-100 Column #6 → 10[b]	24	137	10	2.74×10^3	10	1.3	9	1.2	7	1	2,108	2,280	2,740	9.9	2.3	0.5	0.05

a = Dialysate of ethanol precipitate; octanol used in resuspension.
b = Bacterial control already subtracted.

TABLE XI. AFFINITY PURIFICATION OF AGGLUTININ FROM DARK RED KIDNEY

Prep	Volume (mls)	Corrected+ Volume (mls)	Units per 0.5mls	Total Units	Protein (µg/ml)	Total Protein (mg)	Hexose (µg/ml)	Total Hexose (mg)	Uronic (µg/ml)	Total Ur. Ac. (mg)
CONTROL										
SDW	75	131	0	0	0	0	0	0	0	0
Used SPNT	72	121	0	0	38	5	6	0.7	2	0.2
Cell Wash	368	672	0	0	13	9	2	1	1	0.7
6B-100 Column #6 → 8	29	61	0	0	9	0.3	1	0.06	4	0.2
DARK RED KIDNEY										
Dialysate	75	131	100	2.62×10^4	940	123	400	52	182	24
Used SPNT	69	121	20	4.84×10^3	570	69	460	56	126	15
Cell Wash	383	672	1	1.34×10^3	16	11	6	4	2	1.3
6B-100 Column #6 → 8	30	61	10	1.22×10^3	22	1	3	0.18	5	0.3

+ = Corrected Volume relates this Table to Table V. This Table is essentially the raw data for the Affinity Step section in Table V.

TABLE XII. AFFINITY PURIFICATION OF AGGLUTININ FROM WILKIN (SOYBEAN)

Prep	Volume (mls)	Corrected+ Volume (mls)	Units per 0.5mls	Total Units	Protein (µg/ml)	Total Protein (mg)	Hexose (µg/ml)	Total Hexose (mg)	Uronic Acid (µg/ml)	Total Ur. Ac. (mg)
CONTROL										
SDW	83	333	0	0	0	0	0	0	0	0
Used SPNT	79	289	0	0	17	5	4	1	2	0.6
Cell Wash	283	1144	0	0	12	14	4	5	1	1
6B-100 Column #6 → 10	24	137	0	0	5	0.7	2	0.3	0	0
WILKIN										
Dialysate	83	333	20	1.33×10^4	670	223	390	130	200	67
Used SPNT	72	289	20	1.16×10^4	470	136	138	40	130	38
Cell Wash	285	1144	1	2.29×10^3	19	22	8	9	2	2
6B-100 Column #6 → 10	24	137	10	2.74×10^3	15	2	11	1.5	7	1

+ = Corrected Volume relates this Table to Table X. This Table is essentially the raw data for the Affinity Step section in Table X.

B. Characterization

Agglutinin from any of the legume sources, when chromatographed on Sepharose® 6B-100, eluted in the void volume of the column. Since the exclusion limit of Sepharose® 6B-100 is 4×10^6 daltons, the agglutinin is at least 4×10^6 daltons in molecular weight. Agglutinin activity correlated with a protein peak before the major protein peak (Figures 4 through 9).

Colorimetric analyses of pooled active fractions from the Sepharose® 6B-100 column indicated that the agglutinin obtained from each of the sources is composed of protein, hexose and uronic acid. The main component according to concentration is protein. It is suggested that the agglutinin is a glycoprotein. The purest agglutinin preparations, those obtained by the affinity procedure in conjunction with gel filtration on Sepharose® 6B-100 required less than 1 μg each of protein, hexose, and uronic acid to agglutinate 10^8 cells of P. putida. (See Table XIII below.)

TABLE XIII. COMPONENT TO ACTIVITY RATIOS OF AFFINITY-PURIFIED AGGLUTININ PREPARATIONS

Affinity Preparation	μg/unit activity*		
	Protein	Hexose	Uronic Acid
DARK RED KIDNEY	0.8	0.1	0.1
WILKIN	0.5	0.4	0.4

* = Amounts necessary to agglutinate 10^8 cells of Pseudomonas putida.

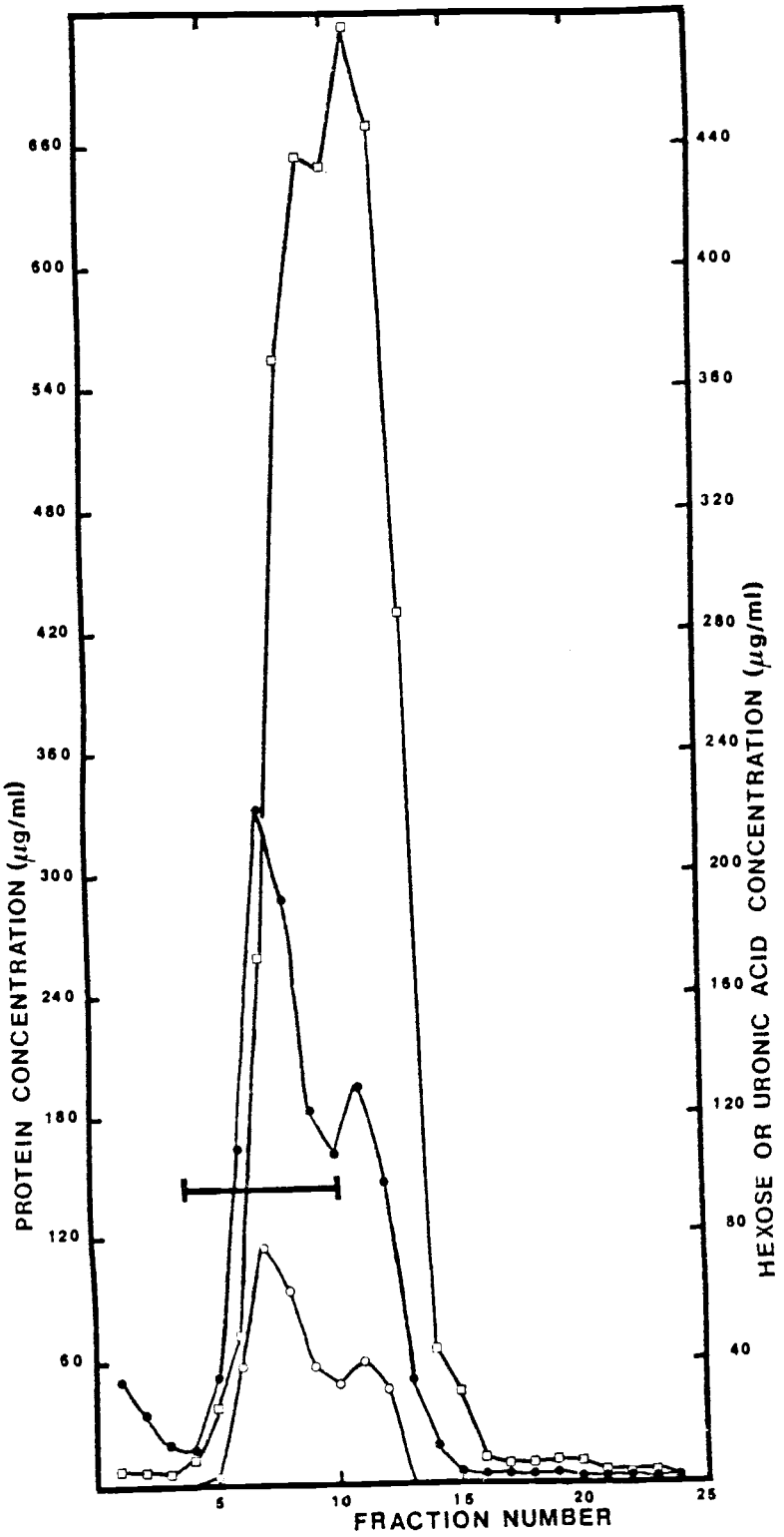

Figure 4. Dark Red Kidney: gel filtration of dialysate. Fifty mls of dialysate concentrated to five mls by rotary evaporation was applied to a Sepharose® 6B-100 column (12.3 cm x 2.3 cm) and eluted with 1mM $MgCl_2$. Protein (□—□), hexose (●—●), and uronic acid (○—○) profiles are shown. Agglutinin activity was found in fractions 4 to 10 denoted by the bar.

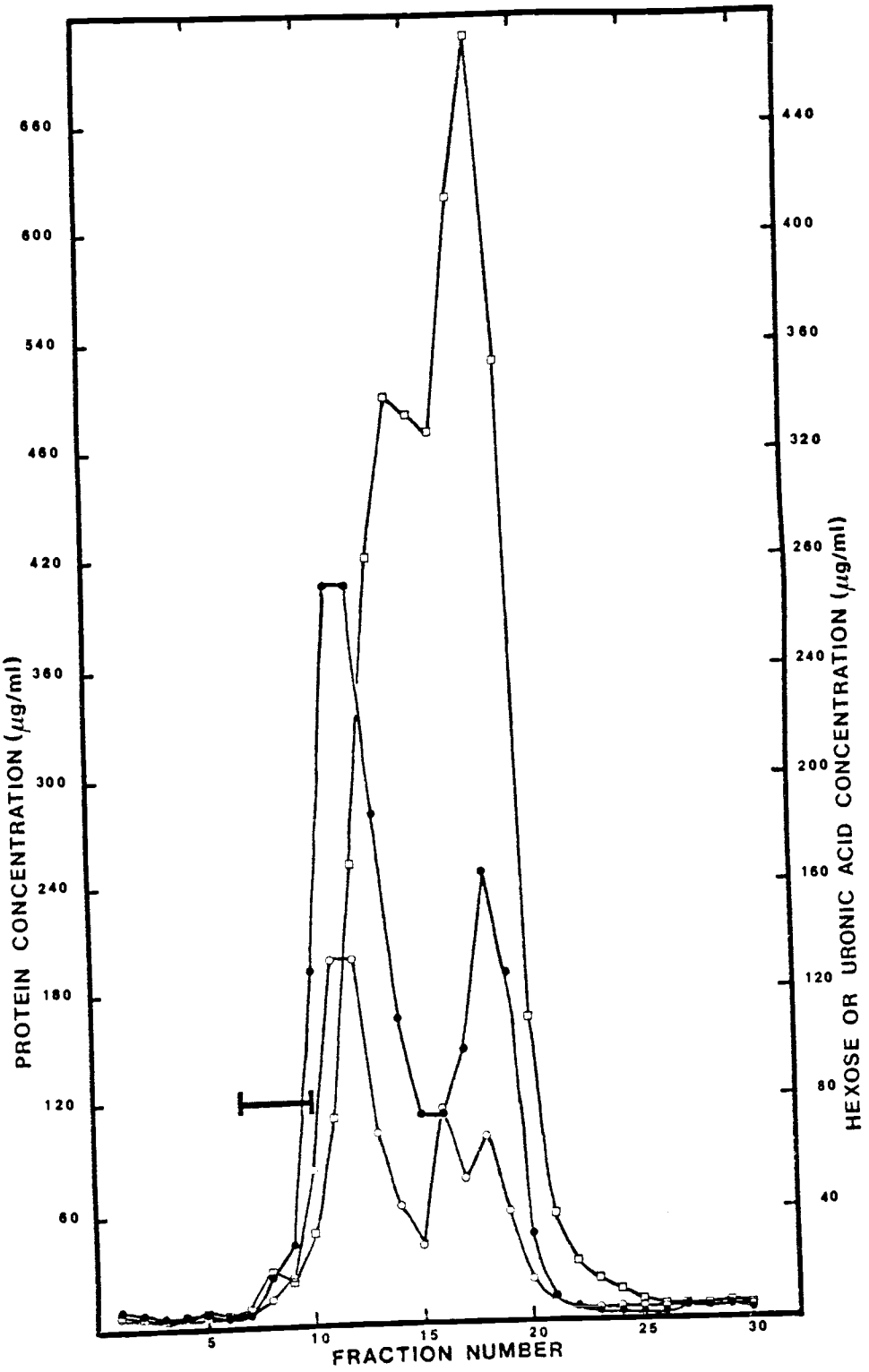

Figure 5. Red Mexican: gel filtration of dialysate.
Fifty mls of dialysate concentrated to five mls by rotary evaporation was applied to a Sepharose® 6B-100 column (23.0 cm x 2.1 cm) and eluted with 1mM $MgCl_2$. Protein (□—□), hexose (●—●), and uronic acid (○—○) profeles are shown. Agglutinin activity was found in fractions 6 to 9 as denoted by the bar.

Figure 6. Fordhook Bush 242: gel filtration of dialysate.
Fifty mls of dialysate concentrated to five mls by rotary evaporation was applied to a Sepharose® 6B-100 column (23.0 cm x 2.1 cm) and eluted with 1mM $MgCl_2$. Protein (□—□), hexose (●—●), and uronic acid (○—○) profiles are shown. Agglutinin activity eluted in fractions 7 to 10 as denoted by the bar.

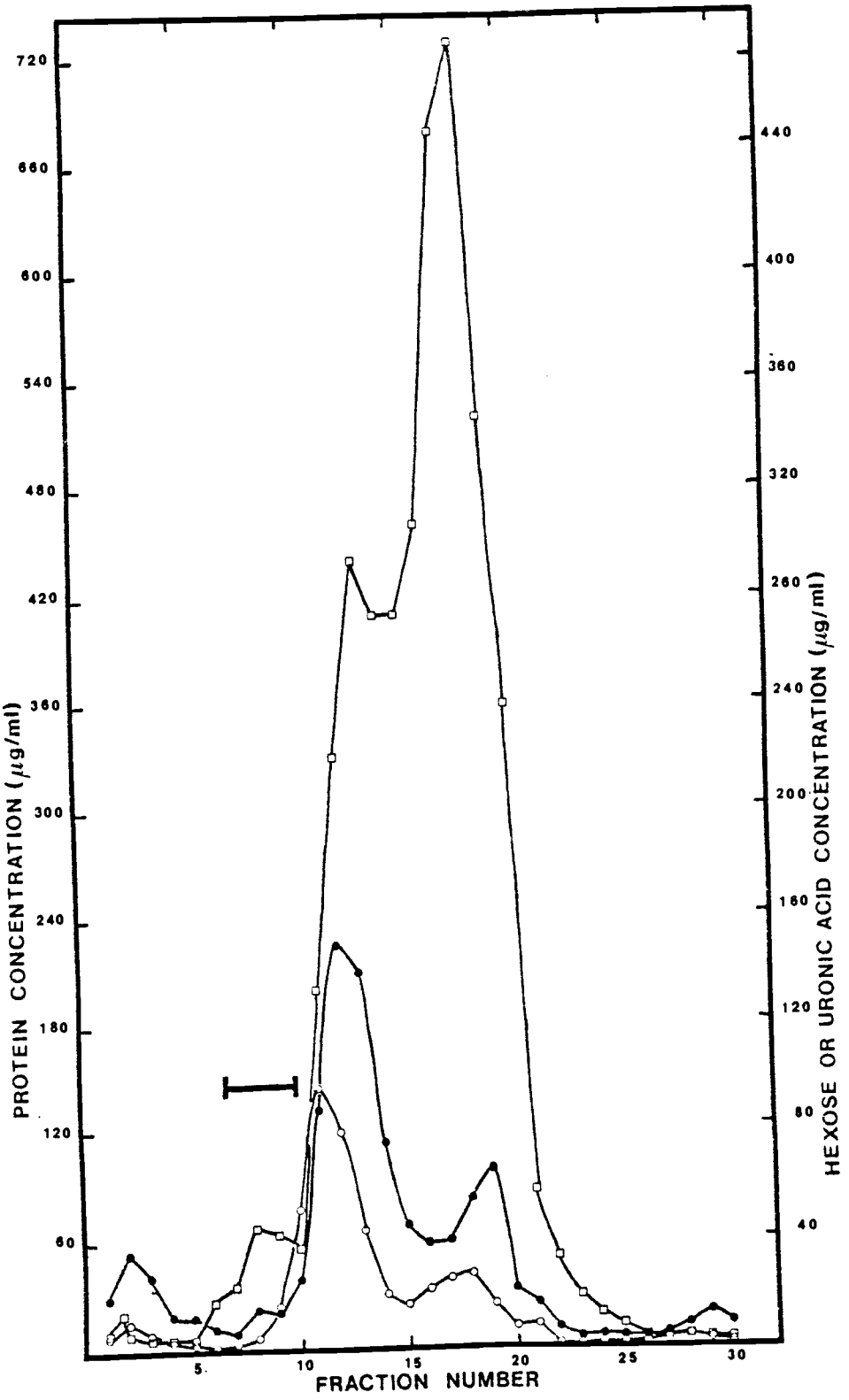
PROTEIN CONCENTRATION (μg/ml)
HEXOSE OR URONIC ACID CONCENTRATION (μg/ml)
FRACTION NUMBER
720
660
600
540
480
420
360
300
240
180
120
60
440
400
360
320
280
240
200
160
120
80
40
5
10
15
20
25
30

Figure 6.

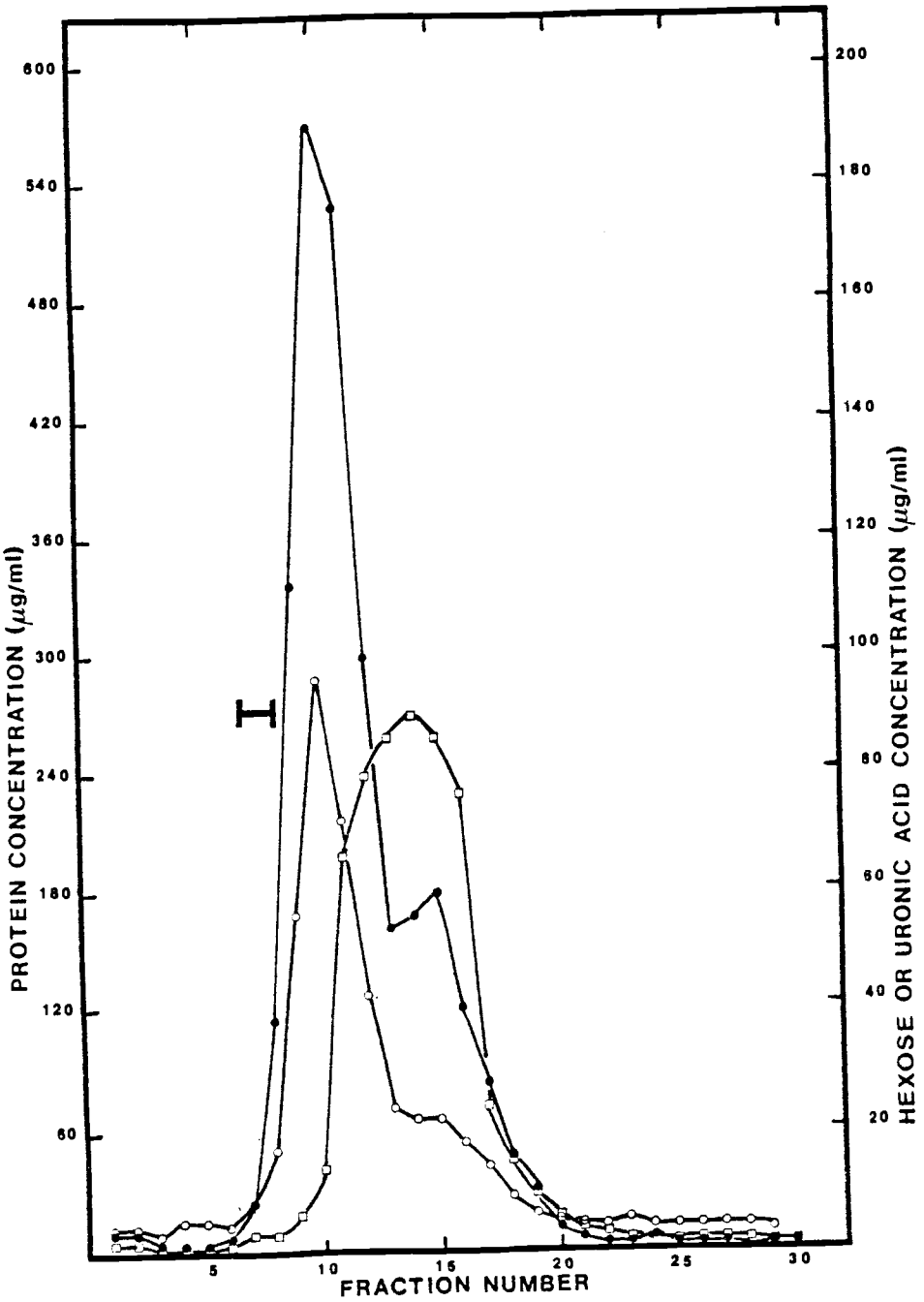

Figure 7. Wilkin: gel filtration of dialysate.
Fifty mls of dialysate concentrated to two mls by rotary evaporation was applied to a Sepharose® 6B-100 column (23.0 cm x 2.1 cm) and eluted with 1mM $MgCl_2$. Protein (□—□), hexose (●—●), and uronic acid (○—○) profiles are shown. The agglutinin activity eluted in fractions 7 and 8 as denoted by the bar.

Figure 8. Dark Red Kidney: gel filtration of affinity purified agglutinin.

Two hundred mls cell wash containing agglutinin (A) or 200 mls control cell wash (B) were concentrated to two mls by rotary evaporation and applied separately to a Sepharose® 6B-100 column (23.0 cm x 2.1 cm) and eluted with 1mM $MgCl_2$. Protein (□—□), hexose (●—●), and uronic acid (○—○) profiles of Dark Red Kidney agglutinin (A) and control (B) columns are shown. The agglutinin eluted in fractions 6 to 8 as denoted by the bar.

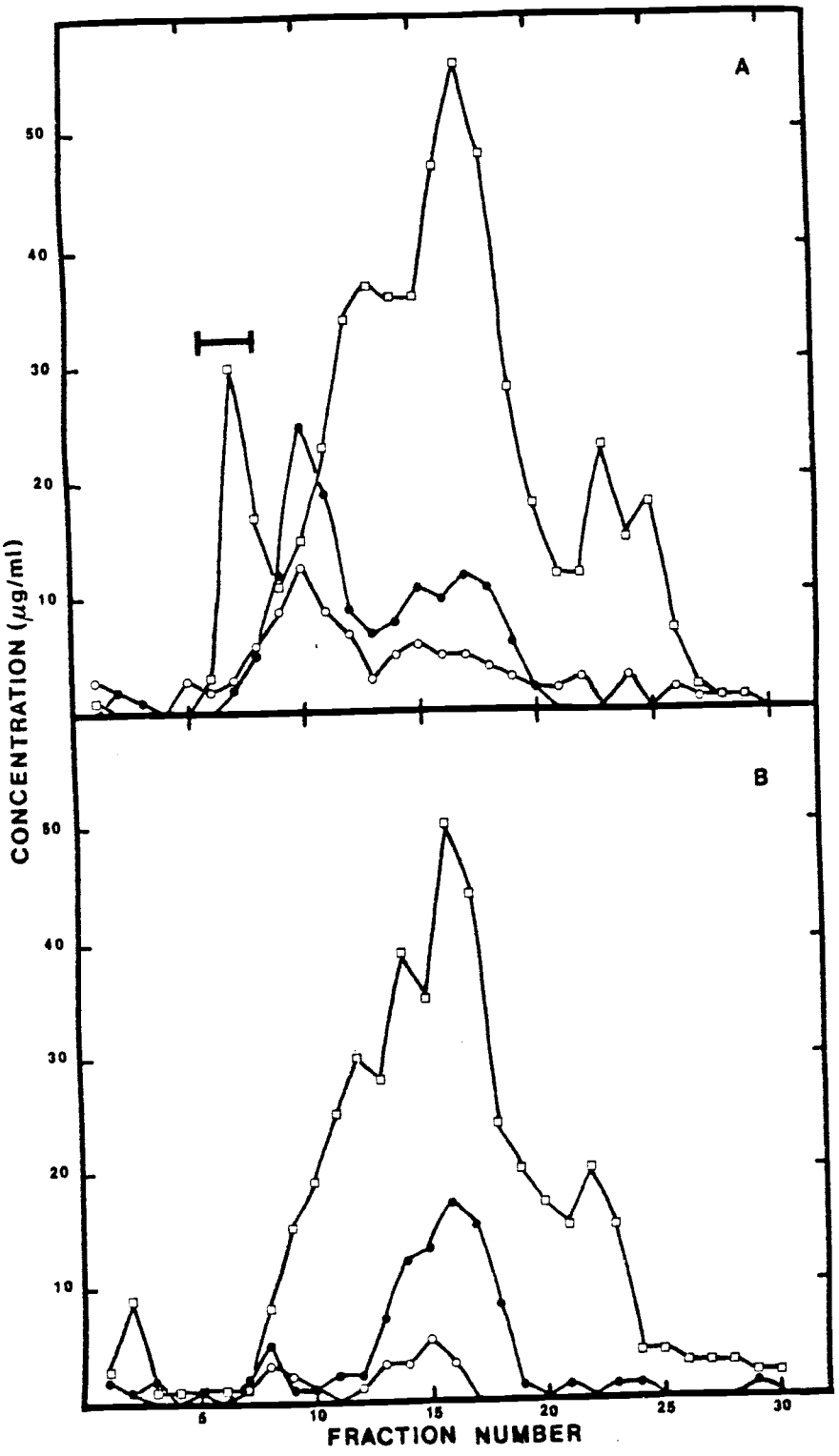
A
B
CONCENTRATION (μg/ml)
FRACTION NUMBER
10
20
30
40
50
5
10
15
20
25
30

Figure 8.

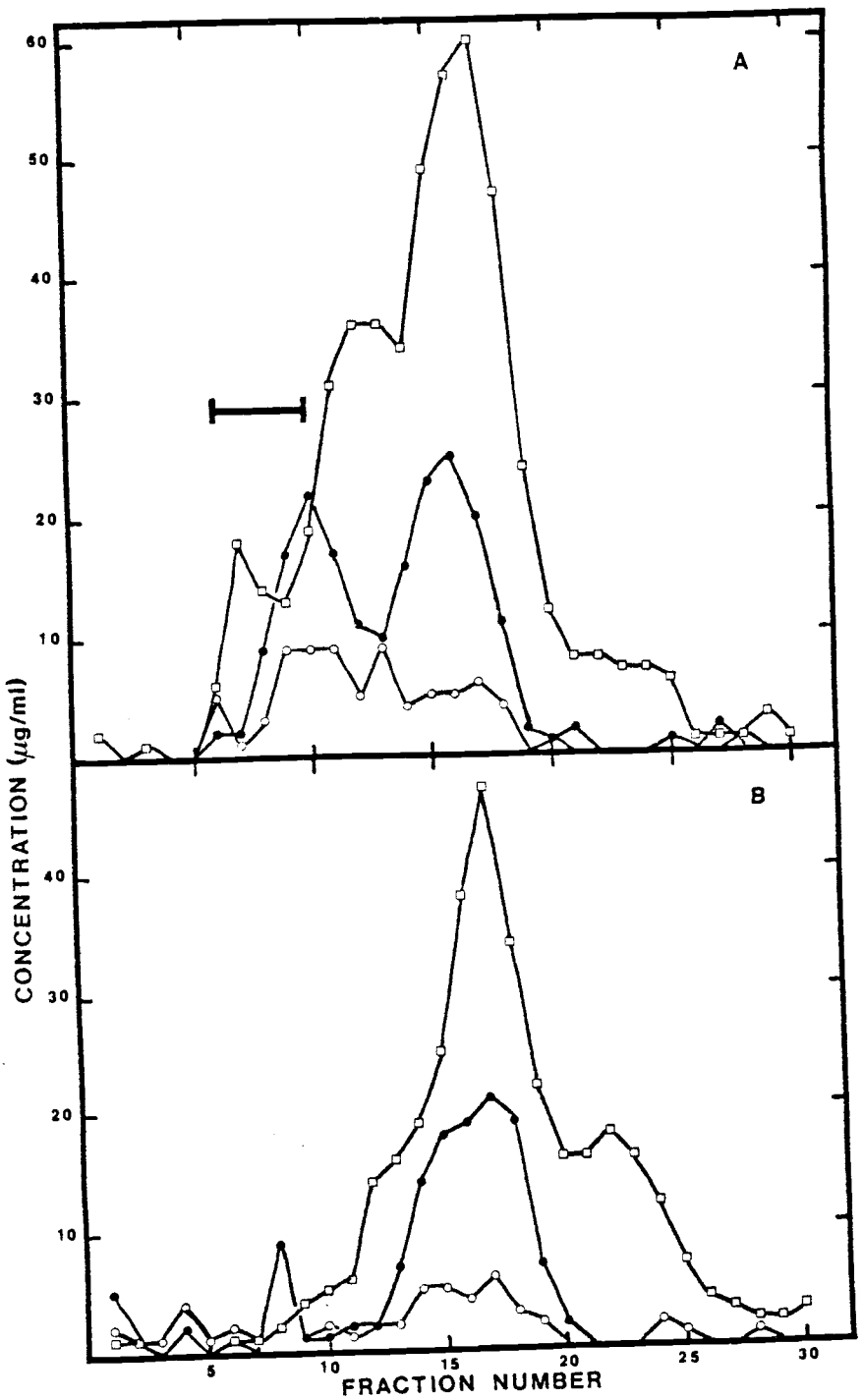

Figure 9. Wilkin: gel filtration of affinity purified agglutinin. Two hundred mls cell wash containing agglutinin (A) or 200 mls control cell wash (B) were concentrated to two mls by rotary evaporation and applied separately to a Sepharose® 6B-100 column (23.0 cm x 2.1 cm) and eluted with 1mM $MgCl_2$. Protein (□—□), hexose (●—●), and uronic acid (○—○) profiles of Wilkin agglutinin (A) and control (B) columns are shown. The agglutinin eluted in fractions 6 to 10 as denoted by the bar.

TABLE XIV. PROPERTIES OF AGGLUTININ FROM BEAN AND SOYBEAN

Activity:	Agglutinin Source			
	Dark Red Kidney	Red Mexican	Fordhook Bush 242	Wilkin
Requires Mg^{++}	Yes	Yes	Yes	Yes
Agglutinate only live bacteria*	Yes	Yes	NA	NA
Periodate sensitive	Yes	Yes	NA	Yes

* = P. putida heat-killed by autoclaving cell suspension for 20 min. at 121°C are not agglutinated.

NA = not assayed.

Agglutinin activity was destroyed by periodate treatment. Periodate is known to cleave glycosidyl residues which have hydroxyl groups on adjacent carbon atoms. The periodate sensitivity suggested that the carbohydrate component of the agglutinin is involved in its activity. Table XIV above lists the agglutinin sources for which this sensitivity has been demonstrated.

The carbohydrate component is composed of neutral sugars and uronic acid according to colorimetric analysis. Gas chromatographic analysis of alditol-acetate derivatives of agglutinin samples from all of the cultivars identified arabinose and galactose as the major neutral sugars present in the carbohydrate component. Agglutinin preparations purified by gel giltration alone had the neutral sugar compositions listed in Table XV. The two cultivars of P. vulgaris and one of P. lunatus contained similar percentages of arabinose and galactose, the average being 35% and 60% respectively. Additionally these preparations contained trace amounts of other neutral sugars. The Dark Red Kidney preparation contained 1% each of fucose, ribose, xylose, and mannose and 4% glucose.

TABLE XV. NEUTRAL SUGAR COMPOSITION OF AGGLUTININ(S) PURIFIED BY GEL FILTRATION ON SEPHAROSE® 6B-100

Agglutinin Source	% Neutral Sugars	
	Arabinose	Galactose
Phaseolus vulgaris		
DARK RED KIDNEY	34	59
RED MEXICAN U. I. 31	36	63
Phaseolus lunatus		
FORDHOOK BUSH 242	36	59
Glycine max		
WILKIN	54	45

TABLE XVI. NEUTRAL SUGAR COMPOSITION OF AGGLUTININ(S) PURIFIED BY THE AFFINITY PROCEDURE AND GEL FILTRATION ON SEPHAROSE® 6B-100

Agglutinin Source	% Neutral Sugars			
	Rhamnose	Ribose	Arabinose	Galactose
Phaseolus vulgaris				
DARK RED KIDNEY	1	8	21	70
Glycine max				
WILKIN	0	1	40	59

The Red Mexican preparation contained 1% fucose, and the Fordhook Bush 242 preparation had 2% rhamnose and 3% mannose. The neutral sugar component of agglutinin purified from Wilkin soybean contained 54% arabinose and 45% galactose with 1% ribose. Agglutinin preparations purified by the affinity procedure and gel filtration had altered percentages. As can be seen in Table XVI, the neutral sugar component of Dark Red Kidney agglutinin was 21% arabinose, 70% galactose, 1% rhamnose, and 8% ribose, and that of Wilkin soybean was 40% arabinose, 59% galactose, and 1% ribose.

The major neutral sugar components of the agglutinin are arabinose and galactose. Arabinose and galactose are the most commonly found carbohydrate constituents in glycoproteins obtained from plants (12). The cell walls of Phaseolus vulgaris and Phaseolus coccineus contain hydroxyproline-rich glycoproteins in which arabinose and galactose are the major constituents of the carbohydrate component (12,47,48); in these proteins the carbohydrate component is linked to the protein through hydroxyproline. A similar situation exists in apple cell walls (31). Cell walls of P. vulgaris also contain a hydroxyproline-poor glycoprotein fraction (11) in which arabinose and galactose are linked through serine or hydroxyproline residues to the protein. The sugars found in the agglutinin are also constituents of major polysaccharide structural components of cell walls (1,18,32). Arabinogalactan chains are crosslinks between xyloglucans and rhamnogalacturonans (1).

C. Determination of Specificity of Agglutinin

In purification agglutinin activity was followed by assaying for agglutination of P. putida. To assess the effectiveness of the

agglutinin against other bacteria a range of isolates was assayed with agglutinin preparations obtained from Dark Red Kidney, Red Mexican and Wilkin. Three different Dark Red Kidney, two Red Mexican, and one Wilkin preparations were used. Each agglutinin preparation was assayed at least eight times against each bacterial isolate, with two cultures of each isolate being used. The bacterial isolates included a number of species of Pseudomonas, encompassing saprophytes, pathogens of hosts other than bean or soybean, and bean and soybean pathogens. Within P. phaseolicola isolates of both Race 1 and Race 2 were examined; the race specificity of these isolates was determined by inoculating them into young plants of Dark Red Kidney and Red Mexican by vacuum infiltration and following symptom development. Dark Red Kidney is susceptible to both races, whereas Red Mexican is susceptible to Race 2 and resistant to Race 1 (45). All of the P. phaseolicola isolates induced systemic symptoms in Dark Red Kidney. Red Mexican was resistant to isolates HB28 and PP. The P. syringae isolate was not pathogenic on these cultivars. These results are summarized in Table XVII. Species of other plant pathogenic genera, ie. Agrobacterium, Erwinia, Corynebacterium, and Xanthomonas and the saprophyte Escherichia coli were also tested.

Results of the agglutination assays are summarized in Tables XVIII and XIX. Only two saprophytic species of Pseudomonas, ie. P. fluorescens and P. putida were agglutinated by the agglutinin preparations from Dark Red Kidney, Red Mexican, and Wilkin. P. fluorescens was weakly agglutinated and in some cases was not agglutinated. P. putida consistently was strongly agglutinated by agglutinin from all three sources. None of the plant pathogenic species of Pseudomonas assayed were agglutinated.

TABLE XVII. REACTION OF *Phaseolus vulgaris* CULTIVARS TO *Pseudomonas phaseolicola* AND *Pseudomonas syringae* ISOLATES

Pseudomonas phaseolicola	*Phaseolus vulgaris*	
	Dark Red Kidney	Red Mexican U. I. 31
HB20	S^s	S
HB28	S^s	R
HB51	NA	NA
G50	S^s	S^s
G51	Ss	S
PP	S^s	S/R
Pseudomonas syringae	R	R

S = susceptible, chlorosis of inoculated monofoliate leaves.

S^s= susceptible and systemic, new trifoliate leaves are chlorotic and stunted.

R = resistant, minimal necrosis to no reaction in inoculated monofoliate leaves.

NA= not assayed.

Likewise, the representative species of other plant pathogenic genera tested, ie. *Agrobacterium*, *Corynebacterium*, *Erwinia*, and *Xanthomonas*, and the saprophyte *Escherichia coli* were not agglutinated. As no isolate of *P. phaseolicola* was agglutinated by either the Dark Red Kidney or the Red Mexican agglutinin preparations, the agglutinin did not distinguish between races of the pathogen. Sterile distilled water controls were especially important inclusions in the bioassay, as at least one of the isolates tested, *P. phaseolicola* G51, exhibited a strong self-agglutination reaction.

TABLE XVIII. AGGLUTINATION OF SAPROPHYTIC AND PHYTOPATHOGENIC BACTERIA BY BEAN AND SOYBEAN AGGLUTININ

Bacterial Isolate	Agglutinin Source		
	Dark Red Kidney[a]	Red Mecican U. I. 31[a]	Wilkin[b]
Agrobacterium radiobacter 84	-	-	-
Agrobacterium rhizogenes A4	-	-	-
Corynebacterium sepedonicum	-	-	-
Erwinia carotovora var. atroseptica SR 6	-	-	-
Erwinia carotovora var. atroseptica SR31	-	-	-
Erwinia carotovora var. carotovora SR 24	-	-	-
Erwinia carotovora var. carotovora SR189	-	-	-
Escherichia coli CSH 125 Sup F thi⁻	-	-	-
Pseudomonas fluorescens	+	+/-	-
Pseudomonas glycinea 100	-	-	-
Pseudomonas lachrymans	-	-	-
Pseudomonas marginalis	-	-	-
Pseudomonas morsprunorum	-	-	-
Pseudomonas phaseolicola (6 isolates)	-	-	-
Pseudomonas putida	+++	+++	+++
Pseudomonas solanacearum	-	-	-
Pseudomonas syringae	-	-	-
Pseudomonas tabaci	-	-	-
Xanthomonas corylina (Rough)	-	-	-

a = Phaseolus vulgaris
b = Glycine max
+ = weak agglutination
+++ = strong agglutination
+/- = variable agglutination
- = no agglutination

Assay conditions as previously described on page 19.

TABLE XIX. AGGLUTINATION OF *Pseudomonas phaseolicola* ISOLATES BY BEAN AGGLUTININ

Pseudomonas phaseolicola	Phaseolus vulgaris	
	Dark Red Kidney	Red Mexican U. I. 31
HB20	-	-
HB28	-	-
HB51	-	-
G50	-	-
PP	-	-
Pseudomonas putida	+++	+++

+++ = strong agglutination
- = no agglutination

Assay conditions as previously described on page 19.

IV. DISCUSSION

An agglutinin has been purified from the leaves of each legume studied. Whether the agglutinins from each of these sources is identical is unknown. The agglutinin is a glycoprotein of at least 4 x 10^6 daltons with a carbohydrate component composed of neutral sugars and uronic acid. The major neutral sugars present are arabinose and galactose. Glycoproteins rich in these two sugars have been isolated from the cell walls of P. vulgaris and P. coccineus by other workers (11,12,47,48). The carbohydrate component of the agglutinin is essential for activity, as activity is destroyed by periodate oxidation. Neutral sugar analysis would suggest that agglutinin from P. vulgaris cv. 'Dark Red Kidney' and cv. 'Red Mexican U. I. 31' and P. lunatus cv. 'Fordhook Bush 242' is similar; that of soybean may be different. The activity specificity of these agglutinin preparations would also support this theory. Dark Red Kidney and Red Mexican agglutinin are active against both P. putida and P. fluorescens and do not distinguish between races of P. phaseolicola, whereas soybean agglutinin is active against only P. putida.

Legume agglutinin agglutinated two Pseudomonas saprophytes P. putida and P. fluorescens, but failed to agglutinate the non-pathogenic soil inhabitant Agrobacterium radiobacter 84, the saprophyte Escherichia coli, and plant pathogenic species in any of the genera assayed.

Recent work on Agrobacterium tumefaciens (34,35,60) may correlate with the failure of the bean agglutinin to clump the Agrobacterium isolates. A. tumefaciens must attach to a wound site in order to initiate a tumor response in bean. Perhaps the bean agglutinin here

purified could be responsible for attachment of A. tumefaciens at a wound site. Isolates of A. tumefaciens should be bioassayed with bean agglutinin. A. tumefaciens and A. radiobacter are closely related (61); the only difference between the two may be the the presence or absence of a plasmid, respectively, which confers virulence.

Within the genus Pseudomonas a pattern of specificity may exist, but more isolates and species of Pseudomonas saprophytes should be assayed. As previously stated the two saprophytic species P. putida and P. fluorescens were agglutinated, while all of the plant pathogenic species were not. According to the proposed nomenclature and classification for plant pathogenic bacteria of Young et al. (61), P. glycinea, P. lachrymans, P. morsprunorum, P. phaseolicola, P. syringae, and P. tabaci are all considered to be pathovars of one species designated P. syringae. Pseudomonas marginalis, P. solanacearum and the two saprophytic species are each still considered to be separate species under this system.

The specificity of legume agglutinin for saprophytic Pseudomonads differs from that observed for isolates of P. solanacearum by potato lectin. Sequeira and Graham (51) assayed 55 virulent and 34 avirulent isolates of P. solanacearum. All of the avirulent isolates were agglutinated by potato lectin, whereas the virulent isolates were not, due to a masking of the lectin receptor site on the bacterial surface by the extracellular polysaccharide slime which virulent isolates of P. solanacearum secrete. Whether or not potato lectin can bind any saprophytic species is unknown at present.

This particular bean agglutinin probably is not responsible for the binding of potential phytopathogenic bacteria to the plant cell

wall in the intercellular spaces of bean leaves; at least it cannot explain the results of electron microscopy studies in bean. Roebuck et al. (46) reported the attachment to the cell wall by fibrillar material of a Race 1 isolate of P. phaseolicola in Red Mexican U. I. 34; this isolate induced a hypersensitive response in this host. However the same Race 1 isolate in the susceptible cultivar Canadian Wonder did not become attached to the cell walls of the leaf mesophyll. Complete encapsulation of the bacteria in Red Mexican, as see in tobacco (50) was not observed. Since the bean agglutinin preparations here purified showed no race specificity, they cannot explain the attachment of an avirulent isolate to the cell wall of a resistant bean cultivar. If the bean agglutinin were present at the cell wall perhaps it could explain the attachment and encapsulation of P. putida or P. fluorescens. There has been one report of encapsulation of P. putida in leaves of Dark Red Kidney by Sing and Schroth (53). However the validity of this report is doubtful, as the workers later attributed "the encapsulation phenomenon" to an artifact of sample preparation, ie. evacuation of water from the sample would cause things present in the intercellular space to be caught up against cell walls. Otherworkers studying the initiation of the hypersensitive response by bacteria in bean found that P. fluorescens induced no response and did not multiply in the intercellular spaces; no EM study of the interaction was made however, so it is unknown if the cells were attached to bean cell walls (39).

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