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Legume Agglutinins That Bind to *Rhizobium meliloti*

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A protein found in seeds and roots of alfalfa (*Medicago sativa*) was implicated in the specificity of the infection process, based on its binding to the symbiont *Rhizobium meliloti*. We found an agglutinin with similar properties in seeds and roots of sweet clover (*Melilotis alba*). The sweet clover differed from alfalfa in nodulation by a mutant strain of *R. meliloti*, but the agglutinins were indistinguishable by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, *Rhizobium* agglutination, and cross-reactivity to antibodies. Similar agglutinins binding *R. meliloti* were found in seeds of legumes from different cross-inoculation groups, including soybean (*Glycine max*), cowpea (*Vigna unguiculata*), pea (*Pisum sativum* L), and mung bean (*Vigna mungo*). The agglutinins from these legumes were recognized by antibodies raised against the agglutinins of alfalfa and sweet clover. Seeds of corn (*Zea mays*) and tomato (*Lycopersicon esculentum*) contained a protein similar to the legume agglutinin, but it did not react with the antibodies. We conclude that the alfalfa agglutinin is representative of a common legume protein and that there is no evidence for its role in specificity or nodule initiation.

A legume is infected by only some strains of rhizobia, and how macrosymbionts and microsymbionts recognize each other is not established. There is evidence that specificity is mediated by lectins, which are plant proteins with binding sites specific for certain sugars or polysaccharides. The model for explaining specificity assumes that a lectin on the root binds a specific polysaccharide on the appropriate *Rhizobium* strain (3). The most studied example is trifoliin A. This protein is found in seeds and roots of *Trifolium repens* and binds *Rhizobium trifolii* (3).

Paau et al. (10) described an agglutinin in alfalfa seed which agglutinates *Rhizobium meliloti* but not other species of rhizobia. The agglutinin failed to agglutinate nonnodulating mutants *R. meliloti* WL113 and WL131, which do not bind to alfalfa roots. Tests with antibodies raised against the seed agglutinin indicated that the agglutinin was present on alfalfa roots but absent from roots of other legumes. Paau et al. (10) suggested two roles for the agglutinin: binding of *Rhizobium* strains to the root and specific recognition.

Recently, the same laboratory reported that the nonnodulating mutants *R. meliloti* WL113 and WL131 do, in fact, agglutinate with the alfalfa agglutinin, although at a lower titer than does the normal nodulating parental strain 102F51 (6). It was found that strain 102F51 is not homogeneous in its agglutinating ability: single-colony isolates gave strains which had high or low agglutinability. With mixed inoculum, the low-agglutinability strains were more competitive and were the dominant strains in nodules. If the mutations producing WL113 and WL131 arose in low-agglutinability cells, then the association of noninfectivity and agglutination is accidental rather than causal. The researchers therefore concluded that the agglutinin might not play a role in *Rhizobium* binding or specificity (6).

Experiments described here confirmed that the agglutinin in alfalfa seeds bound to *R. meliloti* strains at pH 4.0. A similar agglutinin was found in seeds of other legumes. Protein of similar properties was also found in seeds nonleguminous species. The distribution and properties of the agglutinin indicate that if the agglutinin has a role in nodule formation, it is unlikely to confer specificity.

MATERIALS AND METHODS

Rhizobia and seeds. *R. meliloti* 102F51 and mutants derived from it (WL113, WL131, and WL188 [10]) were obtained from W. J. Brill, University of Wisconsin, Madison. *Rhizobium leguminosarum* 128C53 was from the Nitragen Co., Milwaukee, Wis. *Rhizobium* strains were grown on yeast-mannitol broth with shaking at 30°C (13).

Commercial sources provided seeds of alfalfa (*Medicago sativa* cv. Saranac), white-flowered sweet clover (*Melilotis alba*), soybean (*Glycine max* cv. Chippewa), pea (*Pisum sativum* cv. Sparkle), mung bean (*Vigna mungo*), cowpea (*Vigna unguiculata* cv. Calico Crowder), corn (*Zea mays* cv. Golden Bantam), and tomato (*Lycopersicon esculentum* cv. New Yorker). *M. alba* U389 was from H. Gorz, University of Nebraska, Lincoln.

Infectivity tests. Seeds were surface sterilized with concentrated H_2SO_4 for 12 min, followed by rinses with sterile water. Seeds were planted in sterile, aluminum foil-covered 180-ml Dispo bottles (American Scientific Products, Rochester, N.Y.) containing vermiculite and N-free nutrient (7). After 4 days, the bottle was inoculated with 1 ml of a 4-day *Rhizobium* culture in yeast-mannitol broth. Each strain was tested three times. One day later, the foil was removed, and the surface was covered with paraffinized sand (12). The plants were grown at a 16-h–8-h, 26°C-21°C, day-night regimen in a light room. Plants were harvested 25 days after planting and examined for nodulation.

Preparation of agglutinins. Agglutinins from seed were prepared essentially as described by Paau et al. (10). Ground seed was defatted with cold hexane and extracted (1:10, wt/vol) with 10 mM sodium phosphate–0.15 M NaCl (pH 6.8) containing 10 g of sodium ascorbate, 1.2 g of $MgSO_4 7H_2O$, and 20 g of acid-washed polyvinylpyrrolidone per liter. The extract was dialyzed overnight against 100 mM sodium acetate (pH 4) and centrifuged. The supernatant was heated at 80°C for 30 min and centrifuged. Protein was precipitated from the supernatant with 85% saturated $(NH_4)_2SO_4$, sus-

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TABLE 1. Ability of four *Rhizobium* strains to nodulate alfalfa (*M. sativa* cv. Saranac) and two cultivars of sweet clover

R. meliloti strain	Nodulation[a]		
	M. sativa Saranac	*M. alba*	
			U389
102F51	+	+	+
WL188	−[b]	−[b]	−[b]
WL113	−	−	−
WL131	−	+	+

[a] +, Nodules; −, no nodules.
[b] −, Ineffective white swellings.

pended in 0.1 M acetate buffer (pH 4.0), and passed through a Sephadex G-50 column. A single major peak of protein was detected by monitoring the column effluent at 280 nm.

The agglutinins were purified by passing them twice through a Sephadex G-50 column, each time discarding the leading edge of the protein peak (containing higher-molecular-weight protein). These preparations showed only one band after electrophoresis (8) and subsequent silver staining (14).

Antibodies were raised in two New Zealand White rabbits against each of the purified agglutinins from *M. sativa* cv. Saranac and *M. alba* U389. In addition to the standard ration of food pellets (composition unknown), the rabbits received cubes of compressed chopped alfalfa. There was thus a possibility that rabbits became sensitized to legume proteins. As a check for this possibility, we collected preimmune serum as well as an antiserum (against cowpea rhizobia) obtained from a rabbit in the same colony.

Agglutinins in roots. Surface-sterilized seeds were germinated on a plastic screen floating on N-free nutrient. After 1 week, roots were harvested, washed in cold water, frozen in liquid N, and ground with a mortar and pestle. Except for the extraction with cold hexane, agglutinins were prepared as described above.

Cross-reactivity of antigens. An indirect enzyme-linked immunosorbent assay procedure (1) was used to assess the cross-reactivity of the two antigens. The antigens were diluted in sodium carbonate buffer (pH 9.6) consisting of 0.2 g of NaN_3, 1.5 g of N_2CO_3, and 2.93 g of $NaHCO_3$ per liter, added to plate wells (200 μl per well), and incubated at 4°C overnight. Unbound antigens were washed off the plates with three washes of phosphate buffered saline (PBS)–Tween buffer (8 g of NaCl, 0.2 g of KH_2PO_4, 0.2 g of Na_2HPO_4, 0.2 g of KCl, 0.2 g of NaN_3, 0.5 ml of Tween 20 per liter [pH 7.4]). Antiserum diluted 1/500 with PBS-Tween-0.3% bovine serum albumin was added and left for 2 h at room temperature, followed by three washes with PBS-Tween. Alkaline phosphatase-linked goat anti-rabbit antibodies (1/500 in PBS-Tween-bovine serum albumin), 200 μl to each well, was added and incubated for 2 h at room temperature. The wells were then emptied and washed three times with PBS-Tween. Then 200 μl of *p*-nitrophenyl phosphate in glycine buffer (2 mg/ml) was added to each well. After 30 min of incubation at room temperature with shaking, color development was stopped by adding 50 μl of 3 M NaOH per well. Absorbance was measured at 405 nm.

Agglutination assay. Bacteria from 2-day-old cultures were centrifuged at 7,000 × *g*, washed three times in 0.1 M sodium acetate (pH 4.0), and resuspended to an optical density at 660 of 10. The protein concentrations of the agglutinin preparations were measured (2), and the agglutinins were diluted so that their concentrations were similar. Serial 1:2 dilutions of agglutinins were made in 0.1 M sodium acetate (pH 4.0). Agglutinin (25 μl) was added to bacteria (50 μl) in microtitrator plates and agitated for 30 min. The bacteria were then examined microscopically for agglutination and compared with controls mixed with buffer only. To identify possible haptens, carbohydrates (50 mM) were included in some bacterium-agglutinin mixtures.

Agglutinin binding. The agglutinins from alfalfa, sweet clover, pea, and soybean seeds were purified to homogeneity and labeled with fluorescein isothiocyanate (9). A total of 2.5 mg of fluorescein isothiocyanate (isomer 1) was dissolved in 1.0 ml of 0.5 M Na_2CO_3 (pH 9.5) and immediately added to 9.0 ml of agglutinin solution that had been dialyzed against 150 mM NaCl–10 mM sodium phosphate (pH 7.0). The solution was stirred 1 h at room temperature and then incubated at 4°C overnight. Unconjugated fluorescein isothiocyanate was removed by passage through a 60-ml Sephadex G-25 column equilibrated with 150 mM NaCl–10 mM sodium phosphate (pH 7.0). The preparation was centrifuged at 15,000 × *g* for 5 min before assaying it for protein concentration.

Rhizobium cultures were washed once with PBS and three times with 0.1 M sodium acetate (pH 4.0) and suspended in the buffer. The protein content of the suspension was measured (2). Various dilutions of bacteria in 1.0 ml of buffer were mixed with approximately 50 μg of the fluorescein-labeled agglutinin (100 μl) and incubated at room temperature for 1 h on a shaker at 60 rpm. The mixture was centrifuged to precipitate the rhizobia and bound agglutinins. The supernatant (0.9 ml) was added to 1.0 ml of 0.1 M NaOH, and fluorescence was measured at an excitation wavelength of 492 nm and emission wavelength of 517 nm. This gave the amount of labeled agglutinin remaining in solution; the amount adsorbed by the *Rhizobium* cultures was calculated by difference.

RESULTS

Infectivity tests. The infectivity of *Rhizobium* strains on one alfalfa and two sweet clover lines is compared in Table 1. Wild-type strain 102F51 was effective, whereas strain WL113 was ineffective on the legumes tested. The mutant WL188, which Paau et al. reported to be noninfective (10), formed a few small white swellings. Strain WL131 failed to nodulate alfalfa, as Paau et al. reported (10), but formed effective pink nodules on the two sweet clover lines. Strain WL131 reisolated from these sweet clover nodules did not form nodules when retested on alfalfa.

Agglutinins in seeds and roots. Seeds of six leguminous and

TABLE 2. Agglutination titers of legume seed agglutinins with two strains of *R. meliloti*[a]

Source of agglutinin	Strain	Agglutination at dilution of agglutinin:							
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
M. sativa	102F51	+	+	+	+	+	+	−	−
	WL113	+	+	+	−	−	−		
M. alba	102F51	+	+	+	+	+	+	−	−
	WL113	+	+	+	−	−	−		
P. sativum	102F51	+	+	+	+	+	+	+	−
	WL113	+	+	−	−	−	−		

[a] Undiluted agglutinin solutions contained approximately 600 μg of protein per ml. +, Agglutination; −, no agglutination.

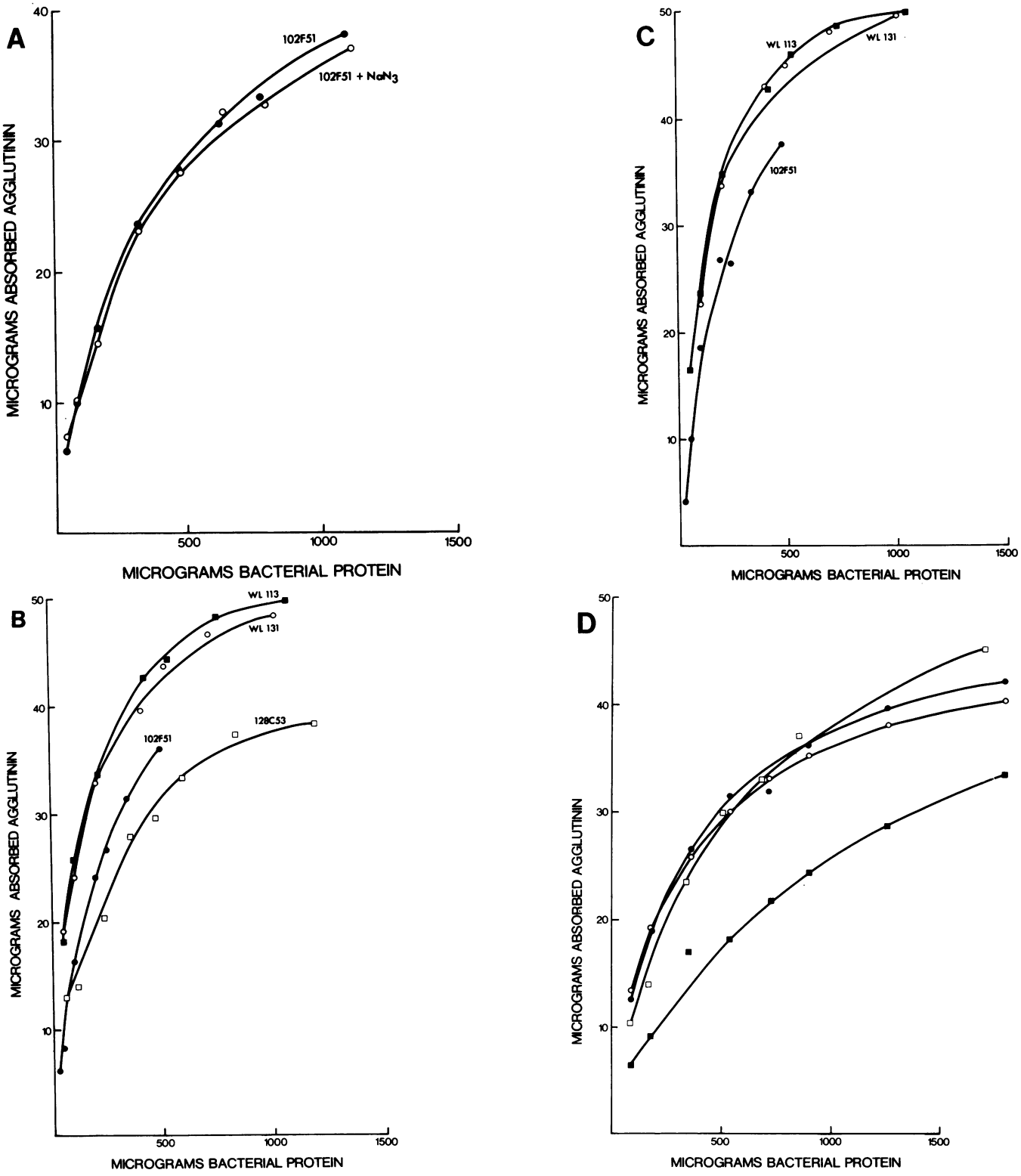
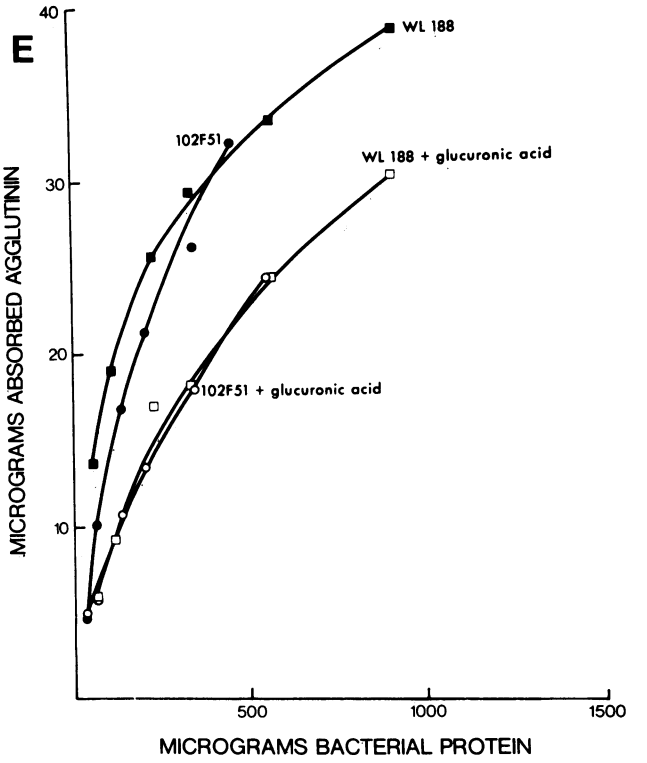
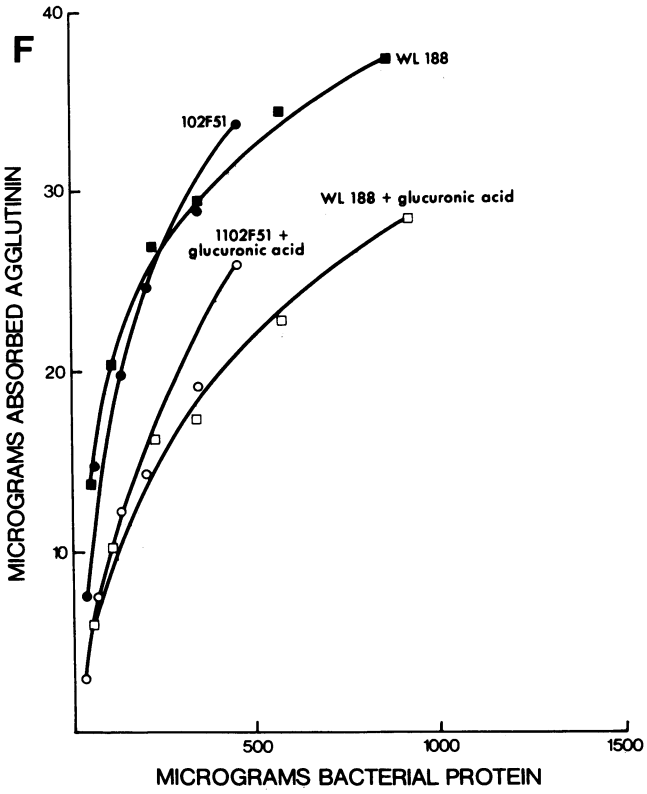


FIG. 1. Adsorption of fluorescein-labeled agglutinins by *Rhizobium* strains. The assay tubes initially contained about 50 μg of protein, and different amounts of rhizobia were added. (A) Binding of alfalfa agglutinin by *R. meliloti* 102F51 without and with 3 mM sodium azide. (B) Binding of alfalfa agglutinin by *R. meliloti* 102F51, WL131, and WL113 and by *R. leguminosarum* 128C53. (C) Binding of sweet clover agglutinin by *R. meliloti* 102F51, WL131, and WL113. (D) Binding by *R. meliloti* 102F51 of agglutinins from alfalfa (○), sweet clover (●), soybean (■), and pea (□). (E) Binding of alfalfa agglutinin by *R. meliloti* 102F51 and WL188 without and with 35 mM glucuronic acid. (F) Binding of sweet clover agglutinin by *R. meliloti* 102F51 and WL188 without and with 35 mM glucuronic acid.





two nonleguminous species were extracted by the method that Paau et al. (10) described for isolating agglutinin from alfalfa. Portions of the protein preparation obtained after passage through a Sephadex G-50 column were run in a 10 or 12% sodium dodecyl sulfate-polyacrylamide gel. In the preparations, the major component was a protein with a mobility slightly greater than that of lysozyme (molecular weight, 14,400) in 12% acrylamide gels and almost identical in 10% gels. Some preparations also contained proteins of higher molecular weight. If in one or two subsequent passages through Sephadex G-50 the leading edge of the protein peak was discarded, only a single band could then be detected. The purified protein preparations from each species were indistinguishable from each other after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein from alfalfa and sweet clover roots was indistinguishable from the protein purified from seeds after sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Cross-reactivity of agglutinins. As little as 0.1 μg of the sweet clover agglutinin gave a strongly positive enzyme-linked immunosorbent assay test (optical density at 405, 1.12) with antiserum raised against the alfalfa agglutinin. Similarly, the alfalfa agglutinin reacted with the antiserum raised against the sweet clover agglutinin (optical density at 405, 0.98). The preimmune sera and the serum raised against cowpea rhizobia did not react with the agglutinins (optical density at 405, <0.034). Thus, the cross-reactivity of the sweet clover and alfalfa agglutinins was not an artifact arising from the rabbit diet.

The purified agglutinins from soybean, cowpea, pea, and mung bean seeds all reacted with antibodies raised against the sweet clover and alfalfa seed agglutinins. The purified agglutinins from alfalfa and sweet clover roots reacted with antibodies raised against the seed proteins. The protein preparations from corn and tomato seed were not reactive.

Agglutination of *R. meliloti*. The seed agglutinins isolated from all the plant species tested could agglutinate *R. meliloti*, as could agglutinins from alfalfa and sweet clover roots. The agglutinins from alfalfa, sweet clover, and pea seeds were very similar in titer (Table 2). Each could agglutinate wild strain 102F51 and, at a lower dilution, mutant WL113. The corn and tomato preparations were much less active than those from legumes (Table 3). They showed slight agglutination of strain 102F51. We never detected agglutination of WL113 (data not shown).

Of the sugars tested, only D-glucuronic acid reduced clumping of strain 102F51 by the alfalfa agglutinins. Agglutination was not prevented by 50 mM D-arabinose, 2-deoxyglucose, D-fructose, D-galactose, D-galacturonic acid, D-gluconic acid, D-glucose, *myo*-inositol, D-lyxose, D-maltose, D-mannitol, D-mannose, α-methyl-D-mannoside, D-raffinose, D-ribose, or D-xylose.

Adsorption of agglutinins by rhizobia. After conjugation with fluorescein isothiocyanate, the agglutinins retained their ability to clump *R. meliloti*. In this experiment, we examined the ability of *Rhizobium* strains to adsorb fluorescein-labeled agglutinins from solution. As the amount of strain 102F51 was increased, more of the labeled alfalfa agglutinin was

TABLE 3. Agglutination titers of seed agglutinins with *R. meliloti* 102F51[a]

Source of agglutinin	Agglutination at dilution of agglutinin:				
	1	1:2	1:4	1:8	1:16
M. sativa	+	+	+	+	−
M. alba	+	+	+	+	−
L. esculentum	+	+	−	−	−
Z. mays	+	−	−	−	−

[a] Undiluted agglutinin solutions contained approximately 120 μg of protein per ml. +, Agglutination; −, no agglutination.

adsorbed (Fig. 1A). This effect was not dependent on cell viability; adsorption was identical in the presence of 3 mM sodium azide (Fig. 1A). Binding of agglutinins was lower at pH 5 (data not shown), and there was no binding at pH 7.

The agglutinin purified from alfalfa seeds was adsorbed not only by the normal nodulating *R. meliloti* 102F51, but also by the nonnodulating mutants WL131 and WL113 and by *R. leguminosarum* 128C53 (Fig. 1B). Nearly identical results were obtained with the agglutinin from sweet clover, which is nodulated by strain WL113, but not by 102F51 and WL131 (Fig. 1C). *R. meliloti* 102F51 bound not only the agglutinins of alfalfa and sweet clover, but also those purified from pea and soybean seeds (Fig. 1D). The adsorption of both alfalfa and sweet clover agglutinins by strains 102F51 and WL188 was reduced by D-glucuronic acid (Fig. 1E and F).

DISCUSSION

We confirmed that alfalfa seeds contain a heat-stable protein that agglutinates *R. meliloti* at pH 4.0 (10). The simple procedure of Paau et al. (10) reproducibly provides a near-homogeneous protein with an apparent molecular weight of about 13,000 in 10% acrylamide gels.

The legume agglutinin is unlike other legume agglutinins which have been described. Trifoliin A, found in seeds and roots of *T. repens*, binds *R. trifolii* but not other *Rhizobium* species. There is strong evidence implicating trifoliin in the specificity of *Rhizobium* binding to clover roots (3). Trifoliin differs from the alfalfa agglutinin in its higher molecular weight (55,000 versus 13,000). The two proteins also differ in hapten-inhibiting agglutination: 2-deoxyglucose blocks agglutination of *R. trifolii* by trifoliin, whereas the alfalfa agglutinin is inhibited by D-glucuronic acid.

The hydroxyproline-rich glycoproteins are a widely distributed class of plant proteins. They are found in cell walls and can agglutinate bacteria or fungal conidia (4). They thus may serve both as a structural component and as a defense against pathogens. The hydroxyproline-rich glycoproteins are insoluble and generally must be extracted from cell walls by acid-ethanol solutions. Molecular weights have been estimated from 55,000 to 110,000. In contrast, the legume agglutinins we studied are readily extracted by neutral aqueous media and have a lower molecular weight.

Agglutination at low pH is found with a lectin extracted from seed of *Glycine max* (soybean) cv. Clark (5). That protein agglutinates *Xanthomonas phaseoli* at pH 4 and, like the alfalfa agglutinin (10), is not a hemagglutinin. The agglutination of *X. phaseoli* is not inhibited by glucuronic acid. Unlike the alfalfa agglutinin, it is composed of two subunits, each about 50,000 molecular weight.

The agglutinin from alfalfa seeds does not react equally with all *Rhizobium* strains. We found, as Handelsman et al. reported (6), that more agglutinin was required to clump the mutants WL113 and WL131 than to agglutinate wild strain 102F51.

We found these binding and agglutinating effects only at pH 4. The requirement for such a low pH to agglutinate *Rhizobium* strains has never been discussed (6, 10), and it is not clear how this experimental condition was determined. We were unable to estimate agglutination of other *Rhizobium* species because the bacteria usually clumped spontaneously at pH 4. Alone among the sugars tried, D-glucuronic acid inhibited both agglutination and binding. If the hapten for the legume agglutinins is a uronic acid, perhaps binding occurs only at a low pH where the carboxyl group is undissociated.

Although it will grow at pH 4, alfalfa is intolerant to nodulation at this low pH value. Rice et al. (11), for example, found that nodulation was reduced at a soil pH below 4.9 and that few *R. meliloti* strains were found in soil of pH 4. We have observed agglutination or binding only at pH 4 to 5. It is therefore difficult to see how the agglutinin could function in binding or specificity at normal soil pH.

That the nonnodulating mutants WL113 and WL131 were not agglutinated was cited as evidence that the agglutinin was responsible for specific recognition (10). However, these strains are now known to bind the agglutinin (6). The fact that strain WL131 nodulates sweet clover but not alfalfa indicates that the mutation involves plant recognition. The fact that the agglutinins in the two plant species are indistinguishable suggests that the mutation in WL131 is not associated with the ability or lack of ability to bind to the agglutinin. Moreover, we find the proteins prepared from other legumes similarly agglutinate all the *R. meliloti* strains tested. The ability of the alfalfa agglutinin to be adsorbed by *R. leguminosarum* further indicates that the role of agglutinin is not associated with specificity.

That *M. alba* contains a similar agglutinin is not unexpected because *Melilotis* and *Medicago* spp. are closely related, and both genera are nodulated by the same cross-inoculation *Rhizobium* group. It was interesting, however, that similar proteins could be isolated from soybean, pea, cowpea, and mung bean seeds; these species are nodulated by other cross-inoculation groups. The proteins must be similar in their physical properties, because they were purified by identical methods. They are all stable to heat and stable at pH 4, and are alike in molecular weight and cross-reactivity to antibodies. It is therefore likely that they have a similar function. Because the agglutinins are from different cross-inoculation groups, that function is not likely to be specific for *Rhizobium* strains.

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