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REGULATION OF THE INTERACTION BETWEEN THE WHITE CLOVER LECTIN, TRIFOLIIN A, AND *RHIZOBIUM TRIFOLII*

By

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ABSTRACT

REGULATION OF THE INTERACTION BETWEEN THE WHITE CLOVER LECTIN, TRIFOLIIN A, AND *RHIZOBIUM TRIFOLII*

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The specific infection of clover root hairs by *Rhizobium trifolii* during the development of a nitrogen-fixing root nodule symbiosis appears to involve the interaction between a clover lectin, trifoliin A, and lectin receptors on the surfaces of the bacterium and root hair. Trifoliin A has been purified from white clover seeds and roots. Trifoliin A consists of one subunit with a molecular weight of 53,000 and varies in aggregate molecular weight between 360,000 and 410,000. The amino acid and glycosyl composition were determined. The optimum pH for bacterial agglutination is 6.5-7.0 and divalent cations are required ($Ca^{++} > Mn^{++} > Mg^{++}$). The specific activity of trifoliin A isolated from white clover roots was 16 times that of the lectin isolated from seeds.

Supplying clover with a fixed nitrogen source reduces both the attachment of *R. trifolii* to root hairs and the level of trifoliin A on the root hair surface. The effect of nitrate supply on trifoliin A synthesis was studied using incorporation of labeled amino acids by heterotrophically growing clover seedlings and quantitative immunoprecipitation. Labeled trifoliin A was detected by fluorography following polyacrylamide gel electrophoresis. While lectin synthesis and excretion into the root exudate was unaffected by excess nitrate, very little lectin in the root exudate was able to bind *R. trifolii*. There was also 10-30 fold less newly synthesized lectin on the root surface of seedlings grown with high levels of nitrate. Examination of total lectin using western blots with antiserum against seed lectin revealed that trifoliin A in seedling shoots, where

little lectin synthesis occurs, was degraded. Newly synthesized root lectin underwent little degradation, but was excreted into the root environment.

The ability of R. trifolii to bind to clover root hairs and interact with trifoliin A changes with culture age, being maximal with 5 day-old plate grown cells. The location of the capsular lectin receptors were generally uniform at 5 and 7 days but mostly polar at 3, 14, and 21 days, even though the capsule itself was uniform after 5 days. The amount and orientation of attachment of R. trifolii to clover root hairs was directly related to the lectin receptor distribution. Chemical analysis of purified capsular polysaccharide (CPS) revealed that the sugar backbone of the CPS remained unchanged, while the degree of acetate and pyruvate substitutions were maximal between 5 and 7 days. These results suggest that trifoliin A interacts with substituted sugars in the CPS, and that the degree of substitution affects the ability of R. trifolii to interact with trifoliin A and bind to root hairs.

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INTRODUCTION

Lectins

Lectins have been defined as sugar-binding proteins or glycoproteins of non-immune origin which agglutinate cells and/or precipitate glycoconjugates (47). They are ubiquitous in nature, being found in bacteria (41), fungi (73), plants (66), and animals (4). Classically, lectins have been detected by their ability to agglutinate red blood cells (hemagglutinins) after they bind to sugars on the cell surface. However, certain lectins are specific for sugars which are not found on the surface of red blood cells (33, 78). The role of lectins has only recently been directly addressed, although their ubiquitious nature suggests a significant role in the organisms in which they are found. The most popular hypothesis currently is that lectins act as regulatory molecules where the lectin is a receptor for a signal molecule, and this specific interaction then elicits a response. This hypothesis has been examined in interactions involving bacterial plant pathogens (87, 92), bacterial pathogens of animals (81), in eucaryotic-eucaryotic interactions involving fungal plant pathogens (39, 44), insects and protozoa (79), and nematode-trapping fungi (73). Additionally, lectins appear to be involved in interactions between symbionts and their hosts, as shown in the Rhizobium-legume (29, 5), lichens (80), and Anabaena-Azolla (61, 68) symbioses. The structure and function of lectins have been extensively reviewed over the past several years (62, 66, 91, 81). Therefore, material most pertinent to this review, namely legume lectins involved in the Rhizobium symbiosis, will be stressed.

Physical and chemical characteristics. Lectins are proteins which are most often glycosylated. The degree of glycosylation can vary dramatically, from the unglycoslated Jack bean lectin, concanavalin A, (66) to the potato lectin, in which sugars comprise 60% of the molecular weight (64). Most legume lectins contain approximately 5 to 10 percent sugar (66). In general, the carbohydrate moiety is not believed to be involved in the sugar-binding interaction since some lectins are not glycosylated and the carbohydrate portion of glycosylated lectins can be gently removed without affecting activity (66). In addition, lectins synthesized in the presence of tunicamycin, which prevents the in vivo glycosylation of proteins, are active (100). The amino acid composition of legume lectins appear to be highly conserved (96). There exists a group of hydroxyproline-rich lectins (up to 42% hydroxyproline) which are quite different in amino acid composition, an example being the potato lectin (64). The N-terminal amino acid sequence has been compared for several legume lectins and found to have up to 80% homology (66, 85, 96). Legume lectins also have a high degree of immunological cross-reactivity, suggesting similarities in protein structure (93).

Other characteristics of legume lectins vary considerably. Lectins vary greatly in subunit molecular weights, from the more typical size of 25,000 to 30,000 up to 265,000 for the lima bean lectin (96). These lectins can also vary greatly in the number and heterogeneity of subunits (66, 77) and sugar specificity. In addition, the glycoconjugate structure of the glycoprotein and the isoelectric point differ among the legume lectins (96). These differences only enhance the attractiveness of the possibility that lectins play a regulatory role.

The synthesis and processing of legume lectins has only recently been examined (17). In most instances, these studies have focused on the synthesis and packaging of lectins as major components in legume seeds (18, 52, 53, 55),

and therefore the cotyledons and developing seeds of mature plants are used. During seed development, the pea lectin is synthesized as a 25,000 mol. wt. polypeptide containing a leader sequence (53). This undergoes both co- and post-translational modification to give the 6,000 and 17,000 molecular weight subunits. In the pea cotyledon, a 23,000 mol. wt. precursor is synthesized in the rough endoplasmic reticulum and transported through the lumen of the endoplasmic reticulum to the protein bodies, where it is processed to the 6,000 and 17,000 m.w. subunits (52). Glycosylation appears to take place in the Golgi apparatus, through which the proteins pass on the way to the protein bodies (17). In bean cotyledons, the major hemagglutinin is synthesized in the endoplasmic reticulum (18). There is also evidence that this lectin is processed from a 243 amino acid polypeptide to a 227 amino acid polypeptide (55). It has been suggested that lectins may serve as seed storage proteins in addition to their regulatory functions (91).

Pertinent to this review are recent studies involving lectins in legume roots and root exudate (23, 35, 42, 43, 45, 46, 90). Goldberg et al. (46) have found soybean lectin transcripts in the roots of soybean seedlings, even in lines which lacked lectin in the seed (83). The gene for the seed lectin was found to contain an insertion which prevented translation of the lectin, although this insertion was not apparent in the root lectin. This suggests that there are different genes encoding the seed and root lectin which are presumably under different regulatory control. However, the seed and root lectins appear similar in structure and are antigenically cross-reactive (90).

The affinity of lectins for sugars has been examined by many methods and may involve several mechanisms. These interactions can be due to ionic or hydrophobic (74) interactions which dictate the methodology used to study them. For example, hydrophobic probes have been extensively used to study the

binding site of the lima bean lectin (84), and the ionic strength of the buffer can greatly affect the interaction (74). The study of these interactions have utilized quantitative affinity chromatography (75), differential UV spectroscopy (67), radioactively (69) and fluorescently (20) labeled saccharides and lectins (44), agglutination of bacteria (26, 92), hemagglutination (66), coated latex beads (82), affinity isoelectric focusing (57), equilibrium dialysis (40), and enzyme-linked immunoassays (12, 35).

Trifoliin A. Trifoliin A is a lectin from clover which binds specifically to Rhizobium trifolii (33). It is a glycoprotein, containing approximately 6 µmol reducing sugar/mg protein, with an estimated subunit molecular weight of 50,000 and an isoelectric point of 7.3 (33). Trifoliin A does not agglutinate red blood cells (33), but does agglutinate R. trifolii cells by interacting with the acidic heteropolysaccharide capsule (CPS) (22) and lipopolysaccharide (LPS) (59) of this bacterium. Only 2-deoxy-D-glucose and the ß-isomer of quinovosamine (2-amino-2,6-dideoxy-D-glucose) have been found to be effective haptens (27, 59). While quinovosamine is found in the LPS of R. trifolii (59), 2-deoxyglucose is found in neither the CPS or LPS of this bacterium. The receptor in the CPS is unknown. Trifoliin A has been found in the seed (33), root surface (33), and root exudate (23) of white clover. The role of trifoliin A in the R. trifolii-clover symbiosis has recently been reviewed (28).

The lectin-recognition hypothesis. The infection and nodulation of legumes by rhizobia is a multi-step process. Motility of the bacteria appears to be influential but not essential (71) and chemotaxis may play a role in competition of infective strains for root nodulation (3). Plant root hairs are deformed early in the infection process by substances of bacterial origin, although the nature of these compounds is unknown (101). Rhizobia attach to the root hairs in a two step process (Dazzo, Truchet, Sherwood, Hrabak, Abe, and Pankratz, submitted

for publication). The first step involves a reversible attachment of the bacteria to the root hair and involves the interaction between bacterial surface polysaccharides and plant lectin. This association becomes irreversible following the synthesis of microfibrils which firmly anchor the bacteria to the root hair surface. The Rhizobium penetrates the root hair cell wall, apparently by enzymatic degradation of the wall (14). Not all root hairs are infectible (9). Newly emergent root hairs are most infectible in soybean, cowpea, and alfalfa. In contrast, clover root hairs may become infected at various degrees of maturity and, if fully mature at the time of inoculation, infections are enhanced by root hair branching (9). Once the bacteria penetrate the root hair, they remain confined to the lumen of an infection thread which grows towards the base of the root hair (19). The infection thread then penetrates the outer cortical cells of the root. At the same time, inner cortex cells opposite the xylem radii are stimulated to enlarge and divide in front of the advancing infection thread (63). The bacteria are released from the infection thread into the plant nodule cells, although they are surrounded by an inverted plant membrane which separates them from the host cell cytoplasm. The bacteria differentiate into pleomorphic bacteroids and fix atmospheric nitrogen into ammonia which is excreted into the host cell cyoplasm. The ammonia is assimilated by the plant as a nitrogen source, while, in turn, the plant supplies the bacteroids with a substrate of photosynthate carbon (19).

The infection of legumes by Rhizobium is a specific process. For example, R. trifolii infects clover, but not alfalfa, peas or soybean. Each of these legumes can only be infected by its homologous symbiont, R. meliloti, R. leguminosarum, and R. japonicum, respectively. The possible involvement of lectins in the Rhizobium-legume symbiosis was first recognized when Hamblin and Kent (51) found that bean lectin interacts with R. phaseoli. Subsequently,

soybean lectin was shown to interact specifically with R. japonicum, which infects soybean, but not with other non-homologous rhizobia (11). The specific interaction between Rhizobium and the lectin from their host plants has been shown to occur in clover (26), pea (60), alfalfa (78), and soybean (95). The lectin-recognition hypothesis states that the specificty of infection seen in the Rhizobium-legume symbiosis is due to this specific interaction between a host lectin and surface receptors of the homologous bacterium.

There have been several lines of evidence which seemingly contradict the lectin recognition hypothesis. However, answers to most of these objections have been found. For example, soybean lectin bound to most, but not all of the infective strains of R. japonicum examined by Bohlool and Schmidt (11). It was subsequently found that these strains were able to bind the lectin when grown in the soybean root environment but not on laboratory medium (8). A strong argument against the lectin-recognition hypothesis was the discovery of soybean lines which apparently lacked the seed lectin but were still infectible (83). This inconsistency has been at least partially resolved by the discovery of a separate root lectin in these lines (46).

Studies of the bacterial lectin receptor which specifically binds the legume lectins have also been inconsistent. Soybean lectin is specific for galactose (66), but the CPS of several strains of R. japonicum contain no galactose and do not bind soybean lectin. These strains have a capsule which consists of rhamnose and 4-O-methyl-glucuronic acid (37), and bind to a recently discovered lectin in soybean which recognizes 4-O-methyl glucuronic acid (36). Lastly, and still unresolved, is the argument that the sugar composition of CPS, extracellular polysaccharide (EPS), and LPS within a cross-inoculum group may be different, while that of CPS, EPS, and LPS of non-homologous bacteria can be identical (15, 37, 86, 103). For example, the sugar composition and structure of the acidic

EPS of some strains of R. trifolii, R. phaseoli, and R. leguminosarum are virtually identical (86), while strains of R. meliloti may have only one of two different types of EPS (37). This presents an obvious problem in explaining the specificity of the lectin recognition hypothesis. However, these polysaccharides are also highly substituted, and can contain methyl (69), pyruvate (38), acetate (38), succinate (54), ß-hydroxybutyrate (56), or combinations of the above. For example, the EPS of R. trifolii 0403 contains pyruvate, acetate, and ß-hydroxybutyrate (56). The substitution of a sugar can dramatically alter the lectin-binding ability as is found in the methylation of galactosyl residues of R. japonicum 3I1b110 (69). As the structures of the Rhizobium lectin receptor become better understood, the role of this interaction in specificity should become clearer.

It should be noted that many researchers have found a positive correlation between the presence of polysaccharide and infectability. The reduction in EPS synthesis by mutants of R. japonicum (65) and R. leguminosarum (72) resulted in decreased infectibility and nodulation of their respective host plants. The addition of EPS from a highly infective strain of R. meliloti increased the infectibility of a poorly infective strain (76). R. trifolii auxotrophs which were incapable of nodulating clover synthesized abnormal EPS (16). A spontaneous revertant capable of nodulation also synthesized normal EPS.

Regulation of the Rhizobium-legume interaction

There are at least three levels at which the Rhizobium-lectin interaction can be regulated. First, the plant may be affected in a way which alters the interaction. This is exemplified by the addition of an excess of a fixed nitrogen source to the growth medium of the legume, at which time all levels of its interaction with the symbiont are negatively affected (97). Secondly, the

bacterium can exhibit culture age-dependent changes in the ability to bind lectin. This has been shown with R. japonicum (69), R. leguminosarum (98), and R. trifolii (32, 59). Thirdly, the interaction between a Rhizobium and its host plant can be affected by interactions between the plant and bacterium (8, 31). Each of these different regulatory mechanisms will be discussed.

The nitrate effect. The addition of a fixed nitrogen source has long been known to inhibit the infection and nodulation of legumes (21, 70). Nitrate was found to have no effect on enzymes involved in nitrogen fixation in intact nodules (49, 58). Ammonium inhibited nitrogenase activity in a nodule homogenate, although the inhibition could be reversed by adding pyruvate and an ATP-generating system (89). The nitrate level in the growth medium of white clover affected infection by R. trifolii, nodule development, and reduced growth of existing nodules (99). In an electron microscopic examination of the entire nodulation process of alfalfa, nitrate was found to be deleterious to the symbiosis at each step involving the interaction between plant and bacterium (97).

In clover, it has been found that 15 mM nitrate inhibits the attachment of R. trifolii to root hairs, as well as its infection and nodulation (21). This was not due to a direct interaction between nitrate or the counter ion with the lectin receptor on the plant or the bacterium, or with the lectin itself (24). The response of the plant to nitrate required more than 1 h exposure to nitrate, since the attachment of R. trifolii to root hairs was unchanged when nitrate was included in the medium in a 1 h attachment assay (24). A 12 h incubation of the plant in nitrate did result in a reduction in attachment (21). The effect of nitrate on the distribution of trifoliin A has also been examined. Growth of clover seedlings in 15 mM nitrate resulted in a 15 to 30-fold decrease in lectin in the root exudate when quantitatied by dilution and immunofluorescence microscopy (23). The level of trifoliin A on root hairs decreased four-fold as

determined by quantitative immunofluorescence assay using a fluorimeter (21). These results detected immunoreactive trifoliin A, and therefore gave an indirect measurement of active lectin on the root hair surface and in the root exudate. However, it was not known if the activity of the lectin was altered, since only active lectin would be detected, or if there was less lectin synthesized or transported out of the root. It has been shown that there are alterations in both the clover (25) and pea (34) root cell walls when these seedlings are grown in nitrate, although no morphological changes were observed in pea root hair cell walls when seedlings were grown with or without nitrate (48). This opens the possibility that a lack of plant lectin receptor contributes to the lower apparent level of trifoliin A on the root hair surface.

Bacterial Regulation. Rhizobium synthesizes a wide variety of polysaccharides, including LPS, ß-1,2-, ß-1,3-, and ß-1,4-glucans, and many different acidic heteropolysaccharides (37, 102). The best studied examples of regulation by the bacterium in the interaction between Rhizobium and lectins involved changes in the surface polysaccharides which occur with culture age (32, 59, 69, 98). In CPS and EPS, these changes involve the addition of non-carbohydrate substitutions to a conserved polysaccharide backbone. The CPS of R. japonicum 3I1b110 undergoes a methylation of carbon 4 of a galactosyl residue during stationary phase (69), resulting in a reduction in soybean lectin-binding ability. Growth-phase dependent changes have also been observed with the CPS and LPS of R. trifolii (32, 59). The LPS of R. trifolii binds to trifoliin A as the culture leaves lag phase and again as it enters stationary phase (59). Chemical analysis of purified LPS showed several changes in the glycosyl composition. One compound which increased when the LPS was able to bind trifoliin A was 2-amino-2,6-dideoxyglucose (quinovosamine). The ß-isomer of quinovosamine was found to bind strongly to trifoliin A (59). It has been shown that the genes

coding for quinovosamine in the LPS (88) and the ability to attach to clover root hairs in a hapten-inhibitable manner are located on the (sym)biotic megaplasmid of R. trifolii (105). The CPS of R. trifolii also shows a transient ability to bind clover lectin, with 5 day-old agar grown cells having the greatest affinity (32). The genetic material required for trifoliin A-binding has been transferred to Azotobacter vinelandii by transformation (10) and to Agrobacterium tumefaciens by transfer of the sym plasmid from R. trifolii (F.B. Dazzo, G.T. Truchet, and P. Hooykaas, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K9, p. 178). The pyruvate and O-acetyl substitutions in the EPS of R. trifolii L158 and other fast-growing rhizobia have been shown to change with culture age (13). The ability of these polysaccharides to interact with their homologous lectins was not examined.

Interactions between the host plant and Rhizobium. The third level of regulation, interactions between the host plant and the rhizobial symbiont, is also the least well defined. The first example of such an interaction came with the finding that strains of R. japonicum which could not bind the soybean lectin when grown in pure culture, attained this ability when grown in the soybean root environment (8). It has also been shown recently that the addition of purified soybean lectin to a mutant R. japonicum which shows a delayed pattern of nodulation results in early nodulation (50). The infection of cowpea by its Rhizobium symbiont was also enhanced by preincubation in the root exudate of the host (6). Interestingly, only root exudate from seedling grown in nitrogen-free medium had this stimulatory effect.

This phenomenon appears to have been best characterized in the R. trifolii-clover symbiosis. Several rhizobial products have been found which increase the infectibility of clover root hairs by R. trifolii. Abe et al. (1) isolated a cyclic ß-1,2 glucan with a molecular weight of approximately 16,000

from the periplasmic space of R. trifolii 4S which is able to increase infection. LPS isolated from a late exponential phase culture of R. trifolii was able to dramatically increase infection thread formation at nanogram levels (30). In addition, an oligosaccharide "branching factor" has been partially purified from the seedling exudate of clover inoculated with R. trifolii (94). This appears significant since mature root hairs of clover appear to be more infectible if they have actively growing branches (7). The net result is an increase in the number of infectible sites on the clover root (7). It is possible that these branching factors are the result of enzymes of host origin which degrade the capsule of R. trifolii (31), or the naturally occuring oligosaccharide repeating units with the same structure as the acidic EPS recently detected in the culture medium of some strains of R. meliloti (2, 104). The possibility that oligosaccharides from Rhizobium can stimulate infection in situ has increased with the recent demonstration that oligosaccharides from purified CPS and EPS produced by phage-induce lyases (Hollingsworth, Abe, Sherwood, and Dazzo, submitted for publication) also have the ability to increase infections in clover (Abe, Sherwood, Hollingsworth, and Dazzo, submitted for publication).

List of References

1. Abe, M., A. Amemura, and S. Higashi. 1982. Plant Soil 64:315-324.
2. Amemura, A., M. Hisamatsu, H. Mitani, and T. Harada. 1983. Carbohydr. Res. 114:277-285.
3. Ames, P., and K. Bergman. 1981. J. Bacteriol. 148:728-729.
4. Barondes, S.H. 1984. Science 223:1259-1264.
5. Bauer, W.D. 1981. Ann. Rev. Plant Physiol. 32:407-449.
6. Bhagwat, A.A., and J. Thomas. 1982. Appl. Environ. Microbiol. 43:800-805.
7. Bhuvaneswari, T.V. 1984. pp. 389-396. In Advances in nitrogen fixation research. C. Veeger and W.E. Newton, eds. Nijoff/Junk and Pudoc, the Netherlands.
8. Bhuvaneswari, T.V., and W.D. Bauer. 1978. Plant Physiol. 62:71-74.
9. Bhuvaneswari, T.V., A.A. Bhagwat, and W.D. Bauer. 1981. Plant Physiol. 68:1144-1149.
10. Bishop, P.E., F.B. Dazzo, E.R. Appelbaum, R.J. Maier, and W.J. Brill. 1977. Science 198:938-940.
11. Bohlool, B.B., and E.L. Schmidt. 1974. Science 185:269-271.

12. Borrebaeck, C.A.K., and B. Mattiasson. 1983. Physiol. Plant. 58:29-32.
13. Cadmus, M.C., K.A. Burton, and M.E. Slodki. 1982. Appl. Environ. Microbiol. 44:242-245.
14. Callaham, D.A., and J.G. Torrey. 1981. Can. J. Bot. 59:1647-1664.
15. Carlson, R.W., R.E. Sanders, C. Napoli, and P. Albersheim. 1978. Plant Physiol. 62:912-917.
16. Caviedes, M.A., M. Megias, A.M. Gil, A.J. Palomares, and F. Ruiz-Berraquero. 1982. Arch. Microbiol. 133:20-22.
17. Chrispeels, M.J. 1984. Phil. Trans. R. Soc. Lond. B 304:309-322.
18. Chrispeels, M.J., and R. Bollini. 1982. Plant Physiol. 70:1425-1428.
19. Dart, P. 1977. pp. 367-472. In A treatise on nitrogen fixation. R.W.F. Hardy and W.S. Silver, eds. J. Wiley and Sons, New York.
20. Dazzo, F.B., and W.J. Brill. 1977. Appl. Environ. Microbiol. 33:132-136.
21. Dazzo, F.B., and W.J. Brill. 1978. Plant Physiol. 62:18-21.
22. Dazzo, F.B., and W.J. Brill. 1979. J. Bacteriol. 137:1362-1373.
23. Dazzo, F.B., and E.M. Hrabak. 1981. J. Supramol. Struct. Cell. Biochem. 16:133-138.
24. Dazzo, F.B., and E.M. Hrabak. 1982. Plant Soil 69:259-264.
25. Dazzo, F.B., E.M. Hrabak, M.R. Urbano, J.E. Sherwood, and G.L. Truchet. 1981. pp. 292-295. In Current perspectives in nitrogen fixation. A.H. Gibson and W.E. Newton, eds. Australian Academy of Science, Canberra.
26. Dazzo, F.B., and D.H. Hubbell. 1975. Appl. Microbiol. 30:1017-1033.
27. Dazzo, F.B., C.A. Napoli, and D.H. Hubbell. 1976. Appl. Environ. Microbiol. 32:166-171.
28. Dazzo, F.B., and J.E. Sherwood. 1983. pp. 209-223. In Chemical taxonomy, molecular biology, and function of plant lectins. L.J. Goldstein and M.E. Etzler, eds. A.R. Liss, Inc., New York.
29. Dazzo, F.B., and G.L. Truchet. 1983. J. Membrane Biol. 73:1-16.
30. Dazzo, F.B., G.L. Truchet, and E.M. Hrabak. 1984. p. 413 In Advances in nitrogen fixation research. C. Veeger and W.E. Newton, eds. Nijoff/Junk and Pudoc, the Netherlands.
31. Dazzo, F.B., G.L. Truchet, J.E. Sherwood, E.M. Hrabak, and A.E. Gardiol. 1982. Appl. Environ. Microbiol. 44:478-490.
32. Dazzo, F.B., M.R. Urbano, and W.J. Brill. 1979. Curr. Microbiol. 2:15-20.
33. Dazzo, F.B., W.E. Yanke, and W.J. Brill. 1978. Biochim. Biophys. Acta 539:276-286.
34. Diaz, C., J.W. Kijne, and A. Quispel. 1981. p. 426 In Current perspectives in nitrogen fixation. Gibson, A.H., and W.E. Newton, eds. Australian Academy of Science, Canberra.
35. Diaz, C.L., P. Lems-van Kan, L.A.M. van der Schaal, and J.W. Kijne. 1984. Planta, in press.
36. Dombrink-Kurtzman, M.A., W.E. Dick, K.A. Burton, M.C. Cadmus, and M.E. Slodki. 1983. Biochem. Biophys. Res. Commun. 111:798-803.
37. Dudman, W.F. 1984. pp. 397-404. In Advances in nitrogen fixation research. C. Veeger and W.E. Newton, eds. Nijhoff/Junk and Pudoc, the Netherlands.
38. Dudman, W.F., and M. Heidelberger. 1969. Science 164:954-955.
39. Esquerre-Tugaye, M-T. 1983. pp. 177-185. In Chemical taxonomy, molecular biology, and function of plant lectins. L.J. Goldstein and M.E. Etzler, eds. A.R. Liss, New York.
40. Etzler, M.E., S. Gupta, and C. Borrebaeck. 1981. J. Biol. Chem. 256:2367-2370.
41. Firon, N., L Ofek, and N. Sharon. 1982. Biochem. Biophys. Res. Commun. 105:1426-1432.

42. Gade, W., M.A. Jack, J.B. Dahl, E.L. Schmidt, and F. Wold. 1981. J. Biol. Chem. 256:12905-12910.
43. Gade, W., E.L. Schmidt, and F. Wold. 1983. Planta 158:108-110.
44. Gibson, D.M., S. Stack, K. Krell, and J. House. 1982. Plant Physiol. 70:560-566.
45. Gietl, C., and H. Ziegler. 1979. Naturwissenschaften 66:161-162.
46. Goldberg, R.B., G. Hoschek, and L.O. Vodkin. 1983. Cell 33:465-475.
47. Goldstein, LJ., R.C. Hughes, M. Monsigny, T. Osawa, and N. Sharon. 1980. Nature 285:66.
48. Goosen-de Roo, L., A.M. Mommaas-Kienhuis, and J. W. Kijne. p. 417 In Advances in nitrogen fixation research. C. Veeger and W.E. Newton, eds. Nijhoff/Junk and Pudoc, the Netherlands.
49. Gordon, J.K., V.K. Shah, and W.J. Brill. 1981. J. Bacteriol. 148:884-888.
50. Halverson, L.J., and G. Stacey. 1984. p. 418. In Advances in nitrogen fixation research. C. Veeger and W.E. Newton, eds. Nijoff/Junk and Pudoc, the Netherlands.
51. Hamblin, J. and S.P. Kent. 1974. Nature New Biol. 245:28-30.
52. Higgins, T.J.V., M.J. Chrispeels, P.M. Chandler, and D. Spencer. 1983. J. Biol. Chem. 258:9550-9552.
53. Higgins, T.J.V., P.M. Chandler, G. Zurawski, S.C. Button, and D. Spencer. 1983. J. Biol. Chem. 258:9544-9549.
54. Hisamatsu, M., J. Abe, A. Amemura, and T. Harada. 1980. Agric. Biol. Chem. 44:1049-1055.
55. Hoffman, L.M., Y. Ma, and R.F. Barker. 1982. Nucleic Acids Res. 10:7819-7828.
56. Hollingsworth, R.L, M. Abe, and F.B. Dazzo. 1984. Carbohydr. Res., in press.
57. Horejsi, V., and M. Ticha. 1981. Anal. Biochem. 116:22-26.
58. Houwaard, F. 1978. Appl. Environ. Microbiol. 35:1061-1065.
59. Hrabak, E.M., M.R. Urbano, and F.B. Dazzo. 1981. J. Bacteriol. 148:697-711.
60. Kato, G., Y. Maruyama, and M. Nakamura. 1981. Plant Cell Physiol. 22:759-771.
61. Kobiler, D., A. Cohen-Sharon, and E. Tel-Or. 1981. FEBS Lett. 133:157-160.
62. Lamport, D.T.A. 1980. pp. 501-541. In The biochemistry of plants, Vol.3. J. Preiss, ed., Academic Press, Inc., New York.
63. Law, LJ., Y. Yamamoto, A.J. Mort, and W.D. Bauer. 1982. Planta 154:100-109.
64. Leach, J.E., M.A. Cantrell, and L. Sequiera. 1982. Plant Physiol. 70:1353-1358.
65. Libbenga, K.R., and P.A.A. Harkes. 1973. Planta 114:17-??.
66. Lis, H., and N. Sharon. 1981. pp. 371-447. In The biochemistry of plants, Vol. 6. M. Marcus, ed. Academic Press, New York.
67. Matsumoto, L., A. Jinbo, H. Kitagaki, A.M. Golovtchenko-Matsumoto, and N. Seno. 1980. J. Biochem. 88:1093-1096.
68. Mellor, R.B., G.M. Gadd, P. Rowell, and W.D.P. Stewart. 1981. Biochem. Biophys. Res. Commun. 99:1348-1353.
69. Mort, A.J. and W.D. Bauer. 1980. Plant Physiol. 66:158-163.
70. Munns, D.N. 1968. Plant Soil 29:33-49.
71. Napoli, C., and P. Albersheim. 1980. J. Bacteriol. 141:979-980.
72. Napoli, C., and P. Albersheim. 1980. J. Bacteriol. 141:1454-1456.
73. Nordbring-Hertz, B., E. Friman, P.A. Johansson, and B. Mattiasson. 1981. pp. 43-50. In Lectins-biology, biochemistry, clinical biochemistry, Vol. 1. T.C. Bog-Hansen, ed. W. de Gruyter, Berlin.
74. Ochoa, J-L. 1981. J. Chromatogr. 215:351-360.

75. Oda, Y., K-L Kasai, and S-L Ishii. 1981. J. Biochem. 89:285-296.
76. Olivares, J., E.J. Bedmar, and E. Martinez-Molina. 1984. J. Appl. Bacteriol. 56:389-393.
77. Osborn, T.C., K.A. Ausloos, J.W.S. Brown, and F.A. Bliss. 1983. Plant Sci. Lett. 31:193-203.
78. Paau, A.S., W.T. Leps, and W.J. Brill. 1981. Science 213:1513-1515.
79. Pereira, M.E.A., A.F.B. Andrade, and J.M.C. Ribeiro. 1981. Science 211:597-600.
80. Petit, P. 1982. New Phytol. 91:705-710.
81. Pistole, T.G. 1981. Ann. Rev. Microbiol. 35:85-112.
82. Pongor S., and Z. Riedl. 1983. Anal. Biochem. 129:51-56.
83. Pull, S.P., S.G. Pueppke, T. Hymowitz, and J.H. Orf. 1978. Science 200:1277-1279.
84. Roberts, D.D., and L.J. Goldstein. 1982. J. Biol. Chem. 257:11274-11277.
85. Roberts, D.D., and L.J. Goldstein. 1984. J. Biol. Chem. 259:909-914.
86. Robertson, B.K., P. Aman, A.G. Darvill, M. McNeil, and P. Albersheim. 1981. Plant Physiol. 67:389-400.
87. Romeiro, R., A. Karr, and R. Goodman. 1981. Plant Physiol. 68:772-777.
88. Russa, R., T. Urbanik, E. Kowalczuk, and Z. Lorkiewicz. 1982. FEMS Microbiol. Lett. 13:161-165.
89. Salminen, S.O. 1981. Biochim. Biophys. Acta 658:1-9.
90. Sengupta-Gopalan, C., J.W. Pitas, and T.C. Hall. 1984. p. 427. In Advances in nitrogen fixation research. C. Veeger and W.E. Newton, eds. Nijoff/Junk and Pudoc, the Netherlands.
91. Sequiera, L. 1978. Ann. Rev. Phytopath. 16:453-481.
92. Sequiera, L. and T.C. Graham. 1977. Physiol. Plant Pathol. 11:43-54.
93. Shannon, L.M. 1983. pp. 47-61 In Chemical taxonomy, molecular biology, and function of plant lectins. LJ. Goldstein and M.E. Etzler, eds. A.R. Liss, New York.
94. Solheim, B., and T.V. Bhuvaneswari. 1984. p. 429. In Advances in nitrogen fixation research. C. Veeger and W.E. Newton, eds. Nijoff/Junk and Pudoc, the Netherlands.
95. Stacey, G., A.S. Paau, and W.J. Brill. 1980. Plant Physiol. 66:609-614.
96. Strosberg, A.D., M. Lauwereys, and A. Foriers. 1983. pp. 7-20 In Chemical taxonomy, molecular biology, and function of plant lectins. LJ. Goldstein and M.E. Etzler,eds. A.R. Liss, New York.
97. Truchet, G.L., and F.B. Dazzo. 1982. Planta 154:352-360.
98. van der Schaal, L., J. Kijne, C. Diaz, and F. van Iren. 1984. pp. 531-538. In Lectins, Vol III. T.C. Bog-Hansen and G.A. Spengler, eds., W. de Gruyter and Co., Berlin.
99. Wedderburn, M.E. 1983. Plant Soil 74:83-91.
100. Yamada, H., T. Hirano, T. Miyazaki, A. Takatsuki, and G. Tamura. 1982. J. Biochem. 92:399-406.
101. Yao, P.Y. and J.M. Vincent. 1976. Plant Soil 45:1-16.
102. Zevenhuizen, L.P.T.M. 1981. Antonie van Leeuwenhoek 47:481-497.
103. Zevenhuizen, L.P.T.M., L Scholten-Koerselman, and M.A. Posthumus. 1980. Arch. Microbiol. 125:1-8.
104. Zevenhuizen, L.P.T.M., and A.R.W. van Neerven. 1983. Carbohydr. Res. 118:127-134.
105. Zurkowski. W. 1980. Microbios 27:27-32.

CHAPTER ONE

PROPERTIES OF TRIFOLIIN A, A *RHIZOBIUM TRIFOLII*-BINDING LECTIN FROM WHITE CLOVER

John E. Sherwood and Frank B. Dazzo

Abstract

The physical, chemical, and *Rhizobium trifolii*-binding properties of the clover lectin, trifoliin A, were examined. A modified purification procedure based on ion exchange and gel permeation chromatography was used to purify trifoliin A from clover seeds and roots resulting in significantly higher yields than obtained from previous methods. The molecular weight of the aggregated lectin was 360,000 to 410,000 using gel permeation chromatography, depending on the pH and ionic strength of the buffer. The lectin consisted of a single 53,000 mol wt. subunit, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The seed lectin, which stains positively with periodate-Schiff, contained approximately 7.0% sugar (w/w) with galactose, glucose, arabinose, and xylose found in a 13:2:2:1 molar ratio per 53,000 mol. wt. subunit. Amino acid analysis of trifoliin A from the seeds of two varieties of white clover, and from the roots of one variety showed that the amino acid composition was generally conserved although the concentration of serine was twice as high in the root lectin than in trifoliin A from seeds. A new bacterial agglutination assay using microtiter plates was used to measure lectin binding

activity. The optimal pH for agglutination of R. trifolii was 6.5-7.0. Agglutination activity was eliminated by EDTA, but could be recovered with the addition of calcium, manganese, or magnesium. The specific activity of the purified root lectin was 16 times that of the purified seed lectin. These improved methods make it possible to purify significantly larger quantities of trifoliin A for analysis of this lectin and and its interaction with R. trifolii.

Introduction

Lectins are sugar binding proteins or glycoproteins which agglutinate cells and/or glycoconjugates (16). The physical-chemical properties (15, 20, 21), biosynthesis (4), and function of lectins (4, 15, 21, 24) have been extensively reviewed. One proposed function for legume lectins has been as intermediates in the interaction between legumes and Rhizobium during the attachment and specific infection of legume root hairs by Rhizobium (2, 8). The lectin from white clover, trifoliin A, has been found in clover seeds (10), on the root surface (10), and in the root exudate of clover seedlings (7). The lectin has been purified from seeds and partially characterized (10), but little subsequent work on the physical-chemical properties has been reported for this lectin although a great deal of information has accumulated concerning the bacterial lectin receptors, lipopolysaccharide (17) and capsular polysaccharide (6, 9). We have modified the procedure for the isolation of trifoliin A and for assaying its activity, and have used these procedures to add additional information concerning the lectin in an effort to clarify its possible role in the clover-R. trifolii interaction.

Materials and Methods

Trifoliin A purification. Trifoliin A was purified from Trifolium repens L. cv. Louisiana Nolin and Ladino seeds using a modification of the procedure of Dazzo et al. (10). Hexane-extracted white clover seed meal was extracted in cold 10 mM Tris-HCl buffer, pH 7.4 containing 0.1% Na ascorbate and 0.3 g acid-washed, insoluble polyvinylpyrrolidone per gram of seed meal. The extraction was performed in a mortar with a pestle at 4°C for 3-5 min, passed through 4 layers of cheesecloth and centrifuged at 20,000 x g for 30 min at 4°C. The supernatant was diluted, if necessary, with cold buffer to a conductivity of less than 3.0×10^3 µmhos. The sample was applied directly to a column (2.5 cm x 30 cm) of DEAE-Sephadex (Pharmacia, Piscataway, N.J.) in Tris buffer at 4°C. Approximately 20 ml of buffer was then added to the column to allow elution of the neutral and cationic material before eluting with about 400 ml of the Tris buffer containing 20 mM $MgSO_4$ (conductivity=3.0×10^3 µmhos). The ascorbate and some of the seed protein elutes at this ionic strength. The lectin was eluted with a linear gradient of 20 to 200 mM $MgSO_4$ (100 ml of each), and 5 ml fractions were collected in polypropylene test tubes (Sarstedt, Princeton, NJ). The lectin began to elute at 4.5×10^3 µmhos; the fractions which contained the lectin were determined by non-denaturing polyacrylamide gel electrophoresis (PAGE) using 7% gels (10). Fractions containing the lectin were pooled and concentrated to 4 ml by ultrafiltration on a YM-30 membrane (Amicon Corp., Lexington. MA) at 4°C. This concentrate was applied to an S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) gel permeation column (2.5 cm x 75 cm), eluted at 4°C with M buffer (10) containing 0.2 mM K phosphate buffer with 0.15 M NaCl, 0.5 mM $CaCl_2$-2 H_2O, 0.15 mM $MnCl_2$-4 H_2O, and 0.5 mM $MgSO_4$. One ml fractions were collected in polypropylene tubes and examined by PAGE. Those fractions which contained

trifoliin A were pooled and concentrated, if necessary, by ultrafiltration. The effluent from both columns was continuously monitored at 280 nm with an ISCO UA-5 monitor (ISCO, Lincoln, NE).

The purity of the final product was determined by sodium dodecyl sulfate (SDS)-PAGE (19) stained with Coomassie blue (18). The specificity of antibody prepared against trifoliin A purified by this method was analyzed by Western blot analysis (1) of the each purification fraction using a 1:600 dilution of the antiserum and probing with ^{125}I-protein A (ICN Radiochemicals, Irvine, CA). A more detailed description of this method can be found in Chapter 2.

Trifoliin A was isolated from Ladino root homogenates using the same procedure. Surface-sterilized clover seeds (5) were imbedded in 1% water agar and placed on stainless steel wire screens in sterilized glass petri plates (150 mm x 20 mm) containing a filter paper disc wetted with water. The seedlings were grown into the humid air within the plates for 5 days at 20°C in a plant growth chamber with a 14 h photoperiod. The roots were harvested by shaving the bottom of the screen with a razor blade, homogenized and fractionated as described above to isolate the lectin.

Characterization of trifoliin A. The aggregate size of trifoliin A was estimated by gel permeation chromatography in a 0.9 x 60 cm column of Sepharose 4B (Pharmacia) using 100 mM Tris-HCl with 0.5 M NaCl, pH 7.2 or 10 mM K phosphate buffer with 0.15 M NaCl, pH 7.0 (PBS), and with an S 300 column (2.5 x 75 cm) using M buffer as the eluent. The effluent was continuously monitored at 280 nm. The elution volumes of the purified lectin were compared with those of standards (Pharmacia) having the following molecular weights: blue dextran, 2,000,000; thryoglobulin, 669,000; ferritin, 440,000; catalase, 232,000; aldolase, 158,000; and bovine serum albumin, 66,200.

The subunit molecular weight was estimated from the R_f value in 10%

SDS-PAGE and comparison with proteins in a low molecular weight standard kit (Bio-Rad, Richmond, CA) which included phosphorylase B, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; and soybean trypsin inhibitor, 21,500. The gels were stained with Coomassie blue.

The absorbance spectrum of the purified lectin (1 mg in 1 ml PBS) was performed on a Varian Cary model 219 spectrophotometer, scanning from 240-310 nm.

The glycosylation of trifoliin A was demonstrated by staining the lectin with periodate-Schiff reagent (Sigma Chemical Co., St. Louis, MO) after non-denaturing PAGE. The carbohydrate content of the purified lectin was estimated using the phenol-sulfuric acid assay (11) with glucose as the standard. This was compared with the amount of protein in the sample determined by the Bio-Rad protein assay (Bio-Rad), used according to manufacturers instructions.

Lectin composition. Amino acid composition was determined by hydrolyzing purified trifoliin A from the seeds of T. repens vars. Ladino (80 µg) and Louisiana Nolin (100 µg), and from the roots of Ladino (30 µg) white clovers for 18 h in 2 ml of 6 N HCl at 121° C, 15 psi. Norleucine (0.4 µmol) was added as an internal standard following hydrolysis. The solution was evaporated to dryness under N_2 and redissolved in deionized, distilled water. Amino acid analyses of these hydrolysates were performed by ion exchange chromatography using lithium citrate buffer and post column derivatization with ninhydrin (12).

The glycosyl composition of the lectin was determined by hydrolyzing 1 mg of purified trifoliin A in 2 N trifloroacetic acid at 121°C for 2 h. The sugars were reduced with $NaBH_4$ and the alditol acetate derivatives were prepared using acetic anhydride:pyridine (1:1, v/v) for 45 min at 100°C. Sugars were separated on an OV 225 column (0.25 x 180 cm) in a Varian 3740 gas chromatograph. The temperature program was 160°C for 6 min followed by a

2°C/min rise to 220°C. Peak areas were determined with a Hewlett Packard 3390A Integrator, and the sugars identified and quantified by comparison with the retention times and detector responses of standard alditol acetates.

Bacterial agglutination assay. Bacterial agglutination was performed using Rhizobium trifolii 0403 (Rothamsted Experimental Station, Harpenden, England) grown for 5 d at 30°C on B III agar (5). The cells were removed from the agar in PBS with a bent glass rod, shaken gently, and centrifuged at 2,300 x g for 30 min at 4°C. The cell pellets were washed twice and resuspended in M buffer. This procedure results in a soft pellet of encapsulated cells (see Chapter 3). The suspension was passed through glass wool in a pasteur pipette to remove cell aggregates (5) and adjusted to an $O.D._{660\ nm}$=0.100 (50 klett units with a Klett-Summerson colorimeter with a no. 66 filter). This cells suspension (25 µl) was added to a two-fold dilution series of the lectin (25 µl) and M buffer (25 µl) in polyvinyl chloride microtiter plates with "U"-shaped wells (Dynatech Laboratories, Alexandria, VA). The plates were sealed, incubated at room temperature and examined periodically through a stereomicroscope using indirect lighting to give illuminated cells against a dark background. The maximum titer achieved without autoagglutination was determined by hourly examination of the microtiter plates.

The optimum pH for bacterial agglutination was determined by dialyzing purified trifoliin A against M buffer adjusted to pH values of 6.0, 6.5, 7.0, 7.5, and 8.0. Encapsulated R. trifolii 0403 suspensions were prepared in each of these buffers as described above, and the appropriate buffer was used to make the 2-fold dilutions of the lectin.

Metal cation requirements for bacterial agglutination were determined by dialyzing purified seed trifoliin A overnight against 0.2 mM K phosphate buffer, pH 6.8 containing 1.0 mM EDTA and then against the same buffer without EDTA

for 24 h with several buffer changes. The lectin was mixed with an equal volume of the 0.2 mM phosphate buffer containing 0.2 mM EDTA, 0.2 mM EDTA plus 290 mM NaCl, or 2.0 mM $CaCl_2$, $MnCl_2$, or $MgSO_4$, all with 290 mM NaCl, and incubated for 1 h at room temperature. Lectin dilutions and bacterial suspensions were made in the appropriate buffer at one half the above concentrations and assayed for agglutinating activity.

Immunofluorescence microscopy of 5 d-old encapsulated cells of R. trifolii 0403 heat fixed to microscope slides was performed using the purified seed and root lectin as described previously (10, also see Chapter 3).

Results

Trifoliin A was purified from the seeds of T. repens L. cv. Louisiana Nolin and Ladino, and from the roots of Ladino. The purification procedure (Table 1) recovered 20% and 40% of the activity from the seeds and roots, respectively. The specific activity increased 7.3-fold from the seed and 85-fold from the roots. The purity of the seed lectin was demonstrated by SDS-PAGE (Fig. 1), resulting in a single band of protein with a molecular weight of 53,000. Analysis of the lectin-containing fractions following each step in the purification procedure by western blot analysis with antiserum prepared against the purified seed lectin and probed with ^{125}I-protein A revealed only the 53,000 mol. wt. lectin (Fig. 1), indicating that there were no other cross-reactive proteins in the seed extract and little, if any, proteolysis of the lectin during purification.

The purified trifoliin A from Louisiana Nolin seeds and Ladino roots were both able to bind to R. trifolii and react with the antiserum prepared against seed trifoliin A, as shown by immunofluorescence microscopy (Fig. 2).

The amino acid composition of trifoliin A from Louisiana Nolin and Ladino seeds and from Ladino roots was determined (Table 2). The lectin from all three

Table 1. Purification of trifoliin A.

Fraction	Total protein[a] (mg)	Total activity (agglutinating units)	Specific Activity (units/mg protein)
Seed extract[b]	35.76	819,000	23,000
DEAE Sephadex	9.56	614,000	64,000
S-300	0.96	162,000	169,000
Root extract[c]	14.52	410,000	28,000
DEAE Sephadex	N.D.	N.D.	N.D.
S-300	0.07	167,000	2,385,000

[a]Measured by the Bio-Rad protein assay.

[b]Extracted from 1.0 g of Lousiana nolin seed meal.

[c]Extracted from 25 g fresh weight of excised Ladino roots.

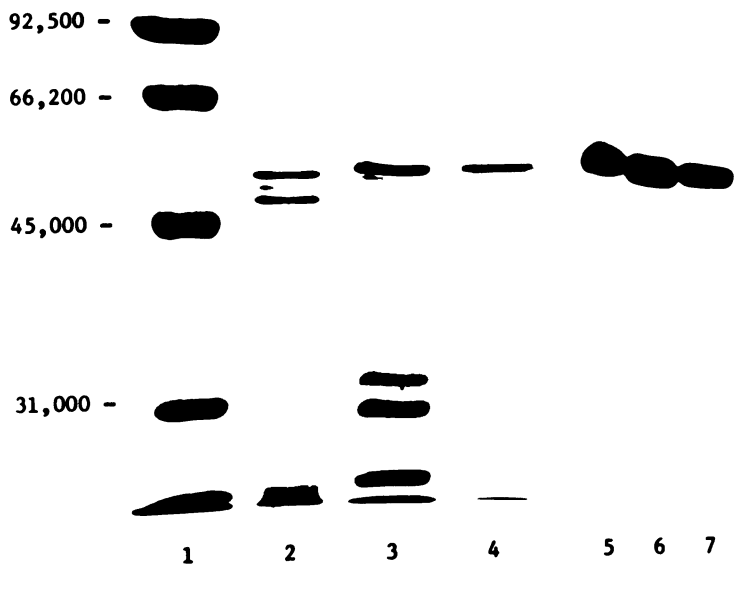

Figure 1. SDS-PAGE analysis of trifoliin A during purification. Molecular weight standards (lane 1), the crude seed extract (lane 2), the DEAE-Sephadex fraction (lane 3) and the S-300 fraction (lane 4) were stained with Coomassie blue. Western blot analysis of the crude seed extract (lane 5), the DEAE-Sephadex fraction (lane 6) and the S-300 fraction (lane 7) revealed only the 53,000 mol. wt. subunit of trifoliin A. Antiserum was prepared against trifoliin A purified from the seed extract.

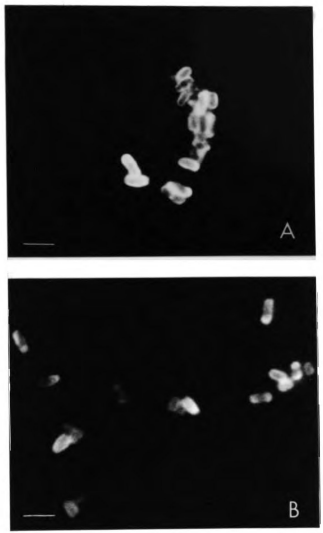

Figure 2. Immunofluorescence microscopy of R. trifolii 0403 treated with (A) purified La. Nolin seed trifoliin A and (B) purified Ladino root trifoliin A. Both lectins were capable of binding to the cells and reacted with antiserum prepared against the purified seed lectin. Bars, 2 μm.

Table 2. Amino acid composition of trifoliin A[a].

Amino acid	Source of purified lectin		
	La. Nolin seeds	Ladino seeds	Ladino roots
asp	9.2	7.8	9.2
thr	5.3	6.4	8.2
ser	3.6	3.7	8.5
glu	12.5	15.4	11.2
gly	9.5	11.1	10.3
pro	0.5	0.7	0.5
ala	11.7	12.7	18.0
val	8.1	7.9	6.9
cys	0	0	0
met	0.2	0.1	0.1
ile	5.1	4.9	4.0
leu	10.7	9.8	7.5
tyr	4.1	1.2	2.1
phe	5.9	5.0	3.5
lys	5.6	5.0	4.0
his	2.3	1.5	1.1
arg	6.6	7.1	4.4
trp	N.D.[b]	N.D.	N.D.
hyp	0	0	0

[a]Expressed as mole percentages.

[b]Not determined.

sources was similar in composition, being high in non-polar (e.g., ala, val, ile, and leu) and acidic amino acids (e.g., asp and glu). The aromatic and basic amino acids were low in concentration, while the sulfur containing amino acids were either present in very low levels (met) or absent (cys). No hydroxyproline was detected. The ultraviolet scan of the purified seed lectin show maxima at 280, 286, and 292 nm. The latter indicated the presence of tryptophan, although the amount of this amino acid in the lectin could not be determined by the procedure used.

Seed trifoliin A stains positively with periodate-Schiff and contains approximately 7% neutral sugar. The glycosyl composition is galactose, glucose, arabinose, and xylose in a molar ratio of 13:2:2:1 (Table 3). There was also a trace of fucose detected, but no N-acetyl-glucosamine or mannose.

The properties of seed trifoliin A are summarized in Table 4. The lectin has an aggregate molecular weight of 360,000 to 410,000 depending on the buffer pH (more aggregated at pH 7.2 than 6.8) and ionic strength (more aggregated in 145 mM NaCl than 500 mM NaCl). The lectin consists of a single subunit with a molecular weight of 53,000. The lectin precipitates at pH values below 6.0 and in acetone concentrations greater than 35% (v/v).

Factors affecting the ability of the lectin to agglutinate R. trifolii 0403 were also examined (Table 4). Agglutination assays performed in M buffer with pH values between 6.0 and 8.0 showed maximal agglutination between pH 6.5 and 7.0. Lectin treated with 1.0 mM EDTA was incapable of agglutinating the bacteria, nor was agglutination ability restored with the addition of 145 mM NaCl. The addition of 1.0 mM Ca^{+2} increased agglutination the greatest amount, followed by 1 mM Mn^{+2}, which was greater than Mg^{+2}. The addition of all three cations, each at 1 mM, gave an agglutination titer equal to that of Mn^{+2} alone. Thus, a modified M buffer containing 1.0 mM $CaCl_2$, pH 6.8, was optimal

Table 3. Carbohydrate composition of trifoliin A from Louisiana Nolin seeds.

Glycosyl component	Carbohydrate content		
	residues/subunit[a]	% of total carbohydrate[b]	% of lectin[a]
galactose	13	74	4.4
glucose	2	11	0.68
arabinose	2	9	0.57
xylose	1	5	0.28

[a]The molecular weight of the lectin subunit is 53,000.

[b]Values were calculated from the total neutral sugar content and the relative amount of each sugar.

Table 4. Summary of trifoliin A properties.

Molecular weight		
Aggregate:	10 mM PBS, pH 7.2	410,000
	100 mM Tris-HCl, 0.5 M NaCl, pH7.2	362,500
	M buffer, pH 6.8[a]	381,300
Subunit:	SDS-PAGE, 10% gel	53,000
Percent glycosylation:		7.0% (w/w)
Isoelectric point:		7.3[b]
Precipitates at:		<pH 6.0
		>35% acetone (v/v)
Bacterial agglutination		
pH optimum:		6.5-7.0
Metal requirements (tested at 1 mM):		$Ca^{+2} > Mn^{+2} > Mg^{+2}$
		Completely inhibited by 0.1 mM EDTA

[a] M buffer is 0.2 mM K phosphate buffer with 145 mM NaCl, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, and 0.15 mM $MnCl_2$, pH 6.8.

[b] From Dazzo et al., 1978.

for bacterial agglutination.

Discussion

The procedures for the purification of trifoliin A and measurement of its activity have been improved from those previously published (10). Use of ion exchange chromatography as the first step permits the isolation of trifoliin A from larger quantities of clover seed meal. Up to 10 g of meal have been extracted and applied to the DEAE-Sephadex column, while only about 1.3 g of seed meal could be processed previously due to volume limitations in the first gel permeation step (10). We have reduced the number of steps in the purification procedure by eliminating the first gel permeation step, a concentration by ultrafiltration, and cation exchange chromatography. The anion exchange column was changed from DEAE-cellulose to DEAE-Sephadex because a portion of trifoliin A binds to cellulose in a hapten (2-deoxyglucose)-elutable manner (unpublished observation). The final gel permeation column was changed from G200 to S300 to allow for a greater flow rate. Trifoliin A also adsorbs non-specifically to glass, polycarbonate, and Amicon PM-10 ultrafiltration membranes (unpublished observations), so these have been substituted with polypropylene tubes for collecting column fractions and for storage, and Amicon YM-30 low protein-binding ultrafiltration membranes. These changes more than doubled the total yield of purified trifoliin A agglutination units recovered from equal amounts of seed meal (E. Hrabak, personal communication).

The determination of the composition of trifoliin A yields information which is useful to study the role of this lectin in the plant, as well as its interaction with R. trifolii. The amino acid composition is similar to other legume lectins (21). The low concentrations of S-containing amino acids in trifoliin A are very typical of legume proteins, and preclude the use of ^{35}S to study synthesis of

the lectin. The relative concentrations of hydrophobic, acidic, basic, and aromatic amino acids in trifoliin A are also typical of legume lectins. Although the amino acid composition of trifoliin A isolated from 3 sources was quite similar, one interesting difference was that the level of serine in the root lectin was more than double that of the seed lectin from either variety of white clover. Serine is one of the few amino acids which forms glycosidic linkages with neutral sugars, usually galactose, in plant glycoprotein (20). The most common glycosidic linkage in plant glycoproteins is glcNac-asn, but glcNac is not found in trifoliin A. The increase in serine in the root lectin may, therefore, permit a greater degree of glycosylation. The yield of the root lectin did not allow for this analysis. Neither UV difference spectroscopy (23) nor fluorescence emmision spectroscopy (13) were successfully applied as methods by which to directly study the interaction between trifoliin A and sugars or isolated surface polysaccharides from R. trifolii 0403. Both of these methods are based on changes in exposed aromatic amino acids when a protein-ligand interaction results in a conformational change of the protein. Such a conformational change may not occur with trifoliin A, or the inability to apply these techniques could be due to the low levels of aromatic amino acids in the lectin. The amount of protein detected by the Lowry method (22), which detects aromatic amino acids and peptide linkages, gives values for purified trifoliin A equal to half of those obtained by the Bio-Rad assay, which is based on the reaction between Coomassie blue and the amino groups of amino acids.

The determination of the sugar composition also yielded interesting results. Many plant glycoproteins, including lectins, contain only mannose and N-acetyl-glucosamine (4, 15, 21), the latter of which, as mentioned above, is usually linked to the protein through an asn residue (20, 21). Other lectins have a more complex sugar composition, including galactose, fucose, arabinose, and

xylose (4, 15), although usually only two or three different glycosyl components are found in any given lectin. Trifoliin A belongs to this latter class of lectin, although its sugar composition appears to be more complex than most lectins. The high level of galactose, which comprises 75% of the total glycosyl component of trifoliin A, raises the possibility of specifically labeling the carbohydrate portion of trifoliin A using radioactive galatose. A similar approach has been used for bean lectin, which contains a high proportion of fucose (3). Additionally, it may be possible to purify trifoliin A using an affinity column with an immobilized lectin with specificity for galactose, such as the lectins from peanut or castor bean (15).

The aggregate size of trifoliin A suggests that there are between 6 and 8 subunits present, depending on the pH and ionic strength of the buffer. Trifoliin A tends to aggregate near its isoelectric point and in low ionic strength buffer. The aggregation of trifoliin A at pH values greater than 7.0 may contribute to the decreased bacterial agglutination activity at these pH values. The aggregates of trifoliin A at pH 7.2 have been detected by transmission electron microscopy following negative staining, and found to be 10 nm in diameter (10).

The modified bacterial agglutination assay using microtiter plates has several advantages over the tube agglutination assay previously described (10). Smaller amounts of lectin and bacteria are required (25 µl vs 200 µl per dilution). The use of microtiter plates also allows for periodic observation of the agglutination without disturbing the cells, so that the maximum agglutination value can be identified. The tube agglutination assay disturbed the agglutinated cells during reading, and therefore could only be observed once. This time was usually set at 4 h, which may not always be the optimal time. In addition, the endpoint titer is more clearly recognized by viewing the microtiter plates under a stereomicroscope than unmagnified, as with the tube assay. These

advantages aided in studying factors which affect bacterial agglutination and, thus, the interaction of the lectin and R. trifolii. Knowing the optimum pH and metal requirements for agglutination are of obvious importance, although it is uncertain whether these factors affect the lectin or the bacterial receptor.

The trifoliin A isolated from clover seeds and roots is active and capable of interacting with R. trifolii, as shown in this study by bacterial agglutination and immunofluorescence microscopy. Trifoliin A isolated from seeds by this procedure was also used for the agglutination inhibition studies in Chapter 3, as well as numerous other studies in our laboratory involving the lectin receptors of R. trifolii, transposon-induced mutants of R. trifolii, and Agrobacterium tumefaciens hybrids containing the symbiotic plasmid from R. trifolii.

Perhaps the most important finding of this study is that the root lectin is much more active (16-fold) than the seed lectin in interacting with R. trifolii. As discussed in Chapter 2, the seed and root lectin in white clover appear to have different roles, with the seed lectin most likely acting as a storage protein which is degraded upon seed germination, while the root lectin is synthesized in the root and excreted into the rhizoplane and rhizosphere where it can interact with R. trifolii. Perhaps the lectins from these two sources may, like those found in soybean seed and root lectins (14), be encoded on different genes and regulated independently. This opens up an exciting area to be investigated in the future.

List of References

1. Battieger, B., W.J. Newhall, and R.B. Jones. 1982. The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. J. Immunol. Meth. 55:297-307.
2. Bohlool, B.B., and E.L. Schmidt. 1974. Lectins: a possible basis for specificity in the Rhizobium-legume root nodule symbiosis. Science 185:269-271.
3. Chrispeels, M.J. 1983. Incorporation of fucose into the carbohydrate moiety of phytohemagglutinin in developing cotyledons of Phaseolus vulgaris. Planta 157:454-461.
4. Chrispeels, M.J. 1984. Biosynthesis, processing and transport of storage proteins and lectins in cotyledons of developing legume seeds. Phil. Trans. R. Soc. Lond. B 304:309-322.
5. Dazzo, F.B. 1982. Leguminous root nodules. pp.431-446. In Methods in microbial ecology. (R.G. Burns and J.H. Slater, eds.) Blackwell Scientific Publications, Oxford.
6. Dazzo, F.B., and W.J. Brill. 1979. Bacterial polysaccharide which binds Rhizobium trifolii to clover root hairs. J. Bacteriol. 137:1362-1373.
7. Dazzo, F.B. and E.M. Hrabak. 1981. Presence of trifoliin A, a Rhizobium-binding lectin, in clover root exudate. J. Supramolec. Struct. Cell. Biochem. 16:133-138.
8. Dazzo, F.B. and D.H. Hubbell. 1975. Cross-reactive antigens and lectin as determinants of symbiotic specificity in the Rhizobium-clover association. Appl. Microbiol. 30:1017-1033.
9. Dazzo, F.B., M.R. Urbano, and W.J. Brill. 1979. Transient appearance of lectin receptors on Rhizobium trifolii. Curr. Micrbiol. 2:15-20.
10. Dazzo, F.B. W.E. Yanke, and W.J. Brill. 1978. Trifoliin: a Rhizobium recognition protein from white clover. Biochim. Biophys. Acta 539:276-286.
11. Dubois, M.K., K.A. Gillies, J.K. Hamilton, P.A. Rebers, and F. Smith. 1956. Colorimetric method for the determination of sugars and related substances. Anal. Biochem. 28:350-356.
12. Erdman, M.D., W.G. Bergen, and C.A. Reddy. 1977. Amino acid profiles and presumptive nutritional assessment of single-cell protein from certain lactobacilli. Appl. Environ. Microbiol. 33:901-905.
13. Glaudemans, C.P.J. and M.E. Jolley. 1980. Method for measuring the affinity between carbohydrate ligands and certain proteins. pp. 145-149. In Methods in carbohydrate chemistry, Vol. VIII. (R.L. Whistler and J.N. BeMiller, eds.) Academic Press, New York.
14. Goldberg, R.B., G. Hoschek, and L.O. Vodkin. 1983. An insertion sequence blocks the expression of a soybean lectin gene. Cell 33:465-475.
15. Goldberg, LJ. and C.E. Hayes. 1978. The lectins: carbohydrate-binding proteins of plants and animals. pp. 127-340. In Advances in carbohydrate chemistry and biochemistry, Vol. 35. (R.S. Tipson and D. Horton, eds.) Academic Press, New York.
16. Goldstein, LJ., R.C. Hughes, M. Monsigny, T. Osawa, and N. Sharon. 1980. What should be called a lectin? Nature (Lond.) 285:66.
17. Hrabak, E.M., M.R. Urbano, and F.B. Dazzo. 1981. Growth-phase dependent immunodeterminants of Rhizobium trifolii lipopolysaccharide which binds trifoliin A, a white clover lectin. J. Bacteriol. 148:697-711.
18. Irie, S., M. Sezaki, and Y. Kato. 1982. A faithful double stain of proteins in polyacrylamide gels with Coomassie blue and silver. Anal. Biochem. 126:350-354.

19. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) 227:680-685.
20. Lamport, D.T.A. 1980. Structure and function of plant glycoproteins. pp. 501-541. In The biochemistry of plants, Vol. 3. (J. Preiss, ed.) Academic Press, New York.
21. Lis, H., and N. Sharon. 1981. Lectins in higher plants. pp. 371-447. In The biochemistry of plants, Vol. 6. (A. Marcus, ed.) Academic Press, New York.
22. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
23. Matsumoto, L, A. Jinbo, H. Kitagaki, A.M. Golovtchenko-Matsumoto, and N. Seno. 1980. Detection of lectin-sugar interaction by ultraviolet difference spectroscopy. J. Biochem. 88: 1093-1096.
24. Sequiera, L. 1978. Lectins and their role in host-pathogen specificty. Ann. Rev. Phytopath. 16:453-481.

CHAPTER TWO

EFFECT OF NITRATE SUPPLY ON THE IN-VIVO SYNTHESIS AND DISTRIBUTION OF TRIFOLIIN A, A *RHIZOBIUM TRIFOLII* BINDING LECTIN, IN *TRIFOLIUM REPENS* SEEDLINGS

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Abstract

In-vivo synthesis of the white clover lectin, trifoliin A, was examined by the incorporation of labeled amino acids into protein during heterotrophic growth of intact *Trifolium repens* L. seedlings. Lectin synthesis was quantified by measuring the level of labeled protein immunoprecipitated from root exudate, from the hapten (2-deoxyglucose) eluate of the roots, and from root and shoot homogenates. The presence of labeled trifoliin A was confirmed by non-denaturing and sodium dodecyl sulfate-polyacrylamide gel electophoresis, followed by fluorography and comparison with trifoliin A standards. In-vivo labeled trifoliin A was detected in seedling root homogenate 2 h after the addition of labeled amino acids and on the root surface by 8 h. Incorporation of labeled amino acids into protein and trifoliin A was greatest with 2 d old seedlings and was greater when the plants were grown continuously in the dark than when they were exposed to a 14 h photoperiod. Significantly more labeled lectin accumulated on the root surface of seedlings grown with 1.5 mM KNO_3 than of seedlings grown either without N or with 15.0 mM KNO_3. The labeled

lectin from the root surface in all nitrate treatments and from the root exudate of the N-free and 1.5 mM KNO_3 grown samples was fully able to bind to R. trifolii. In contrast, only 2% of the immunoprecipitable protein found in the root exudate of 15.0 mM KNO_3-grown seedlings was able to bind to the bacteria. Thus, excess nitrate does not repress the synthesis of trifoliin A in the root, but does affect the distribution and activity of this newly synthesized lectin in a way which reduces its ability to interact with R. trifolii. Western blot analysis detected much more total trifoliin A in the homogenates of shoots than roots. However, greater than 80% of the total labeled protein and 85-90% of the total labeled lectin were found in the root homogenates of 2 d old dark-grown seedlings incubated for 5 h with labeled amino acids. In addition, western blot analysis indicated that the shoot homogenate contained smaller molecular weight peptides which reacted with the specific anti-trifoliin A antibody. These studies suggest that stored trifoliin A in the seed is degraded in the shoots during seedling development, while newly synthesized trifoliin A in the roots is excreted to the root surface and external environment.

Introduction

Lectins are proteins or glycoproteins of non-immune origin that bind to sugars and agglutinate cells (Goldstein et al. 1980). It has been suggested that legume lectins confer specificity in the Rhizobium-legume symbiosis (Bohlool and Schmidt 1974; Dazzo and Hubbell 1975) by interacting with the bacterial symbiont. A lectin from white clover, trifoliin A, binds specifically to R. trifolii via receptors in both the capsular polysaccharide (Dazzo and Brill 1979, Sherwood et al. 1984) and lipopolysaccharide (Hrabak et al. 1981). Trifoliin A is found in clover seeds and on the root surface (Dazzo et al. 1978), as well as in clover root exudate (Dazzo and Hrabak 1981). Several legumes excrete a lectin

from their roots (Dazzo and Hrabak 1981; Gade et al. 1983; Gietl and Ziegler 1979; Kijne et al. 1979), although the synthesis of the lectins by seedling roots was not demonstrated in these reports. It has recently been shown that soybean roots contain lectin mRNA (Goldberg et al. 1983) and that in-vivo labeled soybean lectin can be found in soybean root exudate (Sengupta-Gopalan et al. 1984), including soybean lines which lack the lectin in their seed.

High levels of fixed nitrogen inhibit the infection and nodulation of some legumes by Rhizobium (Munns 1968; Dazzo and Brill 1978; Diaz et al. 1984). The attachment of R. trifolii and the levels of trifoliin A on the root surface of white clover seedlings grown with high nitrate decrease (Dazzo and Brill 1978), as does the level of trifoliin A in the root exudate which can bind to R. trifolii (Dazzo and Hrabak 1981). This reduction in bacterial attachment was not the consequence of a direct interaction between nitrate and trifoliin A or nitrate and the lectin receptor on the root hair surface (Dazzo and Hrabak 1982).

In this report, we demonstrate that trifoliin A is synthesized by intact clover seedling roots and is subsequently found on the root surface and in the root exudate. The effect of nitrate supply on the in-vivo synthesis of trifoliin A by clover roots, its partitioning into root exudate, surface, and internal fractions, and its ability to bind to R. trifolii are also examined.

Materials and methods

Growth of clover seedlings. Commercially obtained Trifolium repens L. cv. Louisiana Nolin seeds were surface-sterilized in acidified 0.1% $HgCl_2$ for 5 min (Dazzo 1982) and germinated for 24 h in the dark on 1% purified agar (Difco, Detroit, Mich., USA) in inverted Petri dishes (100 mm diameter, 15 mm high) at 20°C. The seedlings were then transferred to sterile plastic Petri plates (60 mm diameter, 15 mm high) containing 4 ml of Fahraeus medium (Dazzo 1982) with no

nitrate or supplemented with 1.5 mM or 15.0 mM KNO_3. When comparisons were made between the different nitrate levels, the media were standardized to a constant ionic strength with KCl. Typically, each sample contained 20-30 seedlings. The plates were placed in a plant growth chamber and the seedlings were grown either under a 14 h photoperiod (27,000 lux) with light from incandescent and fluorescent lamps (F48 T12/CW/HO, General Electric, USA) at 20°C, or in the dark by covering the plates with aluminum foil. The growth medium was replaced every other day during experiments requiring more than 2 d incubation.

Since the first phase of root infection by R. trifolii is completed within 8 d (Nutman 1962), the synthesis of trifoliin A by white clover seedlings during this period of development was examined. Seedlings grown in the growth chamber for 2-8 d were transferred to new sterile Petri dishes containing 4 ml Fahraeus medium with the same level of nitrate and either 1.8×10^6 Bq ^3H- or 7.4×10^4 Bq ^{14}C-labeled amino acid mixtures in 0.01N HCl (ICN Pharmaceuticals, Irvine, Cal. USA). The medium was neutralized with 1.0 M sodium-phosphate buffer (pH 7.0) prior to seedling addition.

Following incubation of these seedlings for up to 12 h in a plant growth chamber, the growth medium was removed and considered as the "root exudate" fraction. The seedlings were then washed twice with 4 ml of Fahraeus medium and rinsed in 4 ml of Fahraeus medium containing 30 mM 2-deoxyglucose for 5 min at 100 rpm on a rotary shaker (model G2, New Brunswick Scientific, Edison, N.J., USA). This hapten-eluted rinse was saved as the "root surface" fraction (Dazzo et al. 1978). The seedlings were again washed twice with Fahraeus medium, cut with a razor blade at the crown (just above the root hair region) and weighed. The roots and shoots were homogenized separately in 2 ml of detergent lysis buffer (Witte and Wirth 1979) containing 10 mM sodium

phosphate buffer (pH 7.5), 0.1 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1 mg/ml bovine serum albumin (BSA) with 7-ml scintered glass tissue homogenizers. The homogenates were centrifuged at 15,600g at 4°C and the supernatant was designated the root or shoot homogenate fractions. Samples of the root exudate and root surface fractions were mixed with 10-fold strength detergent lysis buffer to give the same final buffer concentration as the root homogenate fraction.

Precipitation of total protein. Total proteins were precipitated from each sample by adding either cold 50% trichloroacetic acid (TCA) to a final concentration of 5% TCA or four volumes of acetone (Key et al. 1981). The precipitated protein was pelleted at 15,600g for 10 min, washed twice with acetone-ether (1:1, v/v), dissolved in 0.3 ml of SDS sample buffer (Laemmli 1970) and boiled for 15 min.

Immunoprecipitation. Samples in detergent lysis buffer were gently mixed with rabbit anti-trifoliin A immunoglobulin G (IgG) at a final concentration of 0.2 mg/ml (Dazzo et al. 1978) and incubated at 37°C for 2 h and then overnight at 4°C. An excess of goat anti-rabbit IgG Immunobeads (Bio-Rad, Richmond, Cal. USA) (1.0 mg/ml final concentration) was added and the mixture was incubated at 35°C for 4 h with occasional mixing. Titration curves were run to determine the levels of IgG and Immunobeads necessary for the complete removal of labeled trifoliin A from the samples. The beads were centrifuged at 15,600g for 5 min and washed twice in cold PBS. SDS sample buffer was added to the pelleted beads and the mixture was boiled for 15 min.

Sample analysis. Samples of all labeled fractions were mixed in liquid scintillation cocktail (ScintiVerse II, Fisher Scientific Co., Pittsburgh, Penn., USA) and counted in an LS 7000 Liquid Scintillation Counter (Beckman Instruments, Irvine, Cal., USA). A portion of the root exudate fractions (prior to

detergent lysis buffer addition) was analyzed by non-denaturing PAGE (Dazzo et al. 1978), while other portions of all fractions were analyzed by SDS-PAGE (Laemmli 1970) using 10% gels. For fluorography, the gels were treated with EN^{3}HANCE (New England Nuclear, Boston, Mass., USA), dried and exposed to XAR-5 X-ray film (Eastman-Kodak, Rochester, N.Y., USA) at -80°C. Unlabeled standards were run on duplicate gels and stained with Coomassie Blue (Irie et al. 1982).

Trifoliin A in the root and shoot homogenates of 2 day-old dark-grown seedlings was identified by western blot analysis (Burnette 1981). Proteins were transferred electrophoretically from SDS-PAGE gels to pure nitrocellulose membranes (Bio-Rad) using a Trans-blot cell (Bio-Rad) for 16 h at 220 mA. After protein transfer, the membrane was treated with PBS containing 0.05% Tween 20 (Sigma)(PBS-T) for 1 h at 37°C to block non-specific binding of antibody (Batteiger et al. 1982). The membrane was then incubated with rabbit anti-trifoliin A serum diluted 1:600 in PBS-T for 2 h at 37°C, washed twice (0.5 h each) at room temperature with PBS-T and incubated with 1.1 x 10^4 Bq of ^{125}I-protein A (specific activity of 1.0 x 10^5 Bq/µg, ICN Pharmaceuticals) for 1 h at 37°C. The blot was washed twice for 1.0 h at room temperature, dried, and exposed to XAR-5 X-ray film (Eastman Kodak) with a Cronex Lightning-plus intensifier screen (Dupont de Nemours Inc., Wilmington, Del., USA) at -80°C.

To determine the specific activity of the amino acids (cpm/total free amino acids) in the root homogenates of seedlings grown at different nitrate levels, proteins were precipitated in acetone and the supernatant was evaporated to dryness under nitrogen. The residue was redissolved in water to the original volume and counted for internal labeled free amino acids. Total free amino acids in these samples were determined by the colorimetric ninhydrin method of Rosen (1957).

Binding of in-vivo labeled lectin to R. trifolii. Rhizobium trifolii 0403 (obtained from Rothamsted Experimental Station, Harpenden, England) was grown for 5 d on B III agar (Dazzo 1982) at 30°C. The cells were harvested in PBS and placed in a steam cabinet for 1 h to kill the cells and prevent the possible degradation and uptake of labeled peptides during incubation. This process also removed the capsular polysaccharide, which binds to trifoliin A, but would be lost during subsequent centifugation steps. The bacteria were then centrifuged at 13,000g for 15 min at 4°C and washed twice in PBS. The unencapsulated cell pellet was resuspended in PBS and adjusted to 2×10^9 cells/ml.

Labeled root exudate and root surface samples of seedlings grown with different levels of nitrate as described above (triplicate sets of 25 seedlings at each nitrate level) were dialyzed against PBS at 10°C. Lectin-binding lipopolysaccharide (LPS) from R. trifolii 0403 (Hrabak et al. 1981) (0.1 ml of a 20 µg LPS/ml solution), or the same volume of PBS as a control, was added to 1 ml of each sample and the mixture was incubated at 30°C for 1 h. One ml of a suspension of heat-killed R. trifolii 0403 was then added and the mixture was incubated for 2 h at 30°C. The samples were centrifuged, washed twice in PBS at 15,000g for 10 min at 4°C and resuspended in 1 ml of PBS. Treated cells were collected by vacuum filtration on pre-rinsed 0.2-µm Metricel GA-8 membrane filters (Gelman, Ann Arbor, Mich. USA) which were then washed with 15 ml PBS. The membranes were air-dried, placed into scintillation cocktail (ScintiVerse II, Fisher Scientific Co.), and counted. The difference in cpm on the membranes with and without preincubation with lectin-binding LPS was considered to be the amount of active trifoliin A which specifically bound to the bacterial cells. Counts on the membrane in the presence of the LPS were considered background.

Statistical analysis. The counts from the triplicate samples of each nitrate treatment were subjected to a one-way analysis of variance using the F-distribution to test for significant differences in the levels of lectin synthesized and for its ability to bind to R. trifolii 0403. A probability of chance $p<0.05$ was considered indicative of a significant difference.

Results

The in-vivo synthesis of the lectin, trifoliin A, by white clover seedlings was demonstrated by a combination of immunoprecipitation and fluorography. The specificity of the antiserum was demonstrated by western blot analysis, in which one band was seen when the blot of a crude clover seed extract was treated with anti-seed trifoliin A antiserum and ^{125}I-protein A. The major labeled protein which immunoprecipitated from the root exudate and root surface samples was found to co-migrate with trifoliin A purified from clover seeds and roots by non-denaturing and SDS-PAGE (Figure 1). Although labeled protein was immunoprecipitated from the root homogenate samples (10-50 cpm/mg root fresh weight), the radioactivity was too low to be detected by fluorography.

Several variables were examined to optimize the incorporation of labeled amino acids into trifoliin A by the seedlings. The time required to detect in-vivo labeled lectin was determined by analyzing seedlings after 0.5, 1, 2, 4, 6, 8, and 12 h incubation with ^{3}H-labeled amino acids. Labeled immunoprecipitable protein was detected in the root homogenate within 2 h, but labeled lectin was not eluted from the root surface with 2-deoxyglucose until 8 h after amino acid addition. When seedlings were grown under light or dark conditions for 2-8 d and then incubated for 12 h with ^{3}H-labeled amino acids, incorporation of label into immunoprecipitable lectin (Figure 2) and

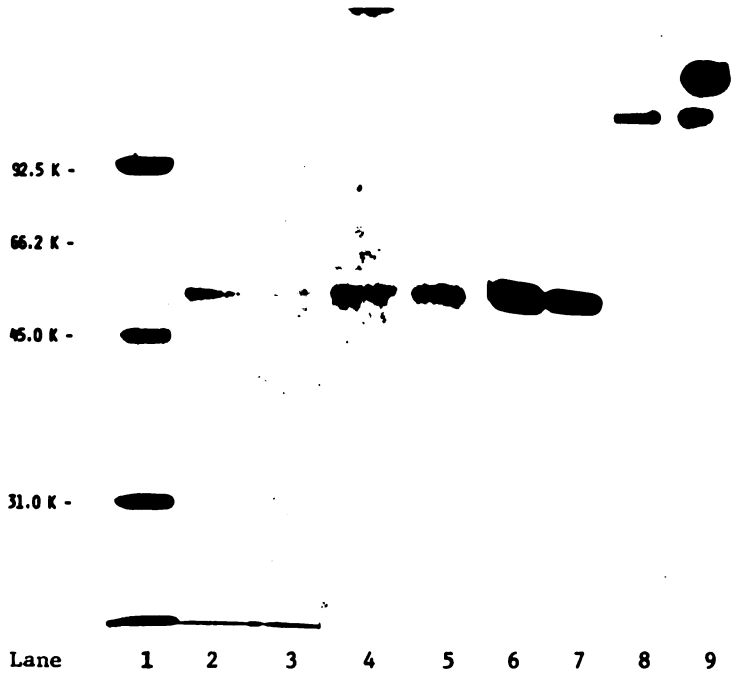

Figure 1. SDS-PAGE analysis of trifoliin A from 2 day-old dark-grown Trifolium repens seedlings. Molecular weight markers (lane 1) and purified lectin from seeds (lane 2) and roots (lane 3) were stained with Coomassie Blue. Tritium-labeled lectin immunoprecipitated from root exudate (lane 4) and root surface (lane 5) were detected by fluorography. The specificity of the anti-trifoliin A antiserum was demonstrated by western blot analysis of a crude white clover seed extract (lane 6) and purified seed trifoliin A (lane 7). Non-denaturing PAGE of purified trifoliin A (lane 8) was stained with Coomassie blue and in-vivo labeled trifoliin A in the acetone precipitate of the root exudate from 2 d-old dark-grown seedlings grown with 1.5 mM KNO_3 (lane 9) was detected by fluorography.

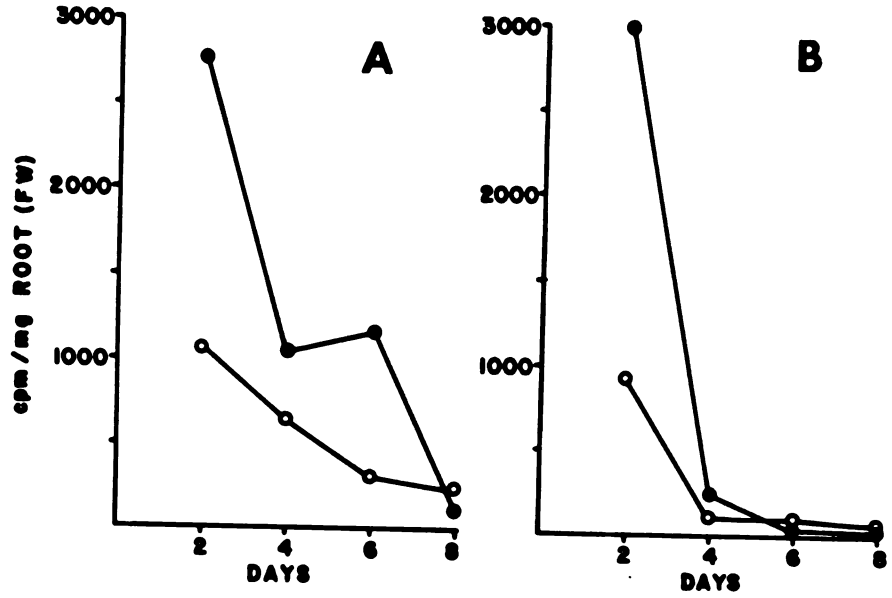

Figure 2. Effect of seedling age on lectin synthesis in the light (O—O) or the dark (●—●). T. repens seedlings of various ages were incubated overnight with ^{3}H-amino acids and the lectin was immunoprecipitated from (A) root exudate and (B) root surface fractions.

TCA-precipitable protein (data not shown) was highest with roots of 2 d-old seedlings and decreased with older seedlings. The immunoprecipitable counts in the root exudate could not be solely attributed to labeled trifoliin A, since there was labeled high molecular weight protein evident when examined by SDS-PAGE-fluorography (Figure 1). This was not a problem with the root surface fractions. The accumulation of labeled lectin on the root surface was greatly affected by the age of the seedling at the time of incubation with labeled amino acids. The amount of labeled lectin was highest on the root surface of 2 day-old seedlings and dropped sharply with older seedlings. In contrast, the level of labeled lectin in the root homogenate remained at a relatively low, constant level over an 8 d period (2-24 cpm/mg root fresh weight). In all cases, the incorporation of labeled amino acids into trifoliin A was greater in seedlings grown completely in the dark than in seedlings exposed to a 14 h photoperiod (Figure 2). The amount of labeled amino acids incorporated into immunoprecipitable protein varied between experiments, but the relative effects of nitrate on the distribution of labeled lectin remained constant (Table 1). Typically, the percentage of the acetone-precipitable labeled protein which immunoprecipitated was 20-30% in the root exudate fraction, 40-50% in the root surface fraction, and 0.1-1.0 % in the root homogenate, regardless of seedling age or growth conditions. The effects of light and seedling age on incorporation of labeled amino acids into protein appeared to reflect the general heterotrophic activity of the seedlings and were not specific for trifoliin A.

The effect of nitrate supply on trifoliin A synthesis was more closely examined with 2 d-old dark-grown seedlings (Table 2). The ^{14}C-label in immunoprecipitable protein from root exudate was not significantly different between the N-free, 1.5mM, and 15.0 mM KNO_3 treatments. The level of labeled

Table 1. Effect of nitrate and growth of T. repens in light or dark on the incorporation of labeled amino acids into trifoliin A.

Experiment	Treatment[a]	Immunoprecipitated protein cpm/mg root fresh weight		
		Root exudate	Root surface	Root homogenate
1	Grown in the dark			
	1.5 mM KNO_3	1241	1115	35
	15.0 mM KNO_3	1415	145	53
2	Grown in the dark			
	1.5 mM KNO_3	1407	1498	11
	15.0 mM KNO_3	1922	851	11
3	Grown in the dark			
	1.5 mM KNO_3	2814	2998	22
	15.0 mM KNO_3	3203	1418	18
	Grown in the light			
	1.5 mM KNO_3	1079	923	10
	15.0 mM KNO_3	2078	537	10

[a]Two day-old seedlings were incubated for the final 12 h with 5.7×10^6 Bq of ^3H-labeled amino acids.

lectin eluted by the hapten from the root surface of seedlings grown with 1.5 mM KNO_3 was significantly higher than from seedlings grown with either no nitrate or 15.0 mM KNO_3. Seedlings grown under N-free conditions had significantly more root surface labeled lectin than did seedlings grown with the highest nitrate level. The amounts of labeled protein from dialyzed root exudate and hapten-eluted root surface samples which specifically bound to R. trifolii (active trifoliin A) were compared to the level of labeled immunoprecipitated protein (total trifoliin A) (Table 2). Only 2% of the labeled immunoprecipitable protein from the root exudate of seedlings grown with 15.0 mM nitrate bound to the bacteria. In contrast, all of the labeled immunoprecipitable trifoliin A in the other samples was active in binding to R. trifolii (Table 2).

The ratio of labeled amino acids to total free amino acids in the roots of 2 day-old dark-grown seedlings was examined to determine if the variations in the level of labeled protein were due to variations in the specific activity of the available internal amino acids. The specific activity of the free amino acids in the root homogenate was not significantly affected by nitrate addition. The specific activity in the root homogenate of seedlings grown without nitrate was 59,200 $\pm$ 4,100 cpm/µmol free amino acid; of 1.5 mM nitrate-grown seedlings, 53,800 $\pm$ 6,000 cpm/µmol free amino acid; and of 15.0 mM nitrate-grown seedlings, 58,000 $\pm$ 7,500 cpm/µmol free amino acids.

Seedlings were cut at the crown (just above the root hair region) following the hapten wash to determine the distribution of labeled total protein and labeled trifoliin A. Approximately 80% of the labeled total protein which precipitated with acetone was found in the roots of seedlings grown with 1.5 mM KNO_3 (1465 $\pm$ 293 cpm/seedling in the root vs. 367 $\pm$ 29 cpm/seedling in the shoot) and 15.0 mM KNO_3 (9195 $\pm$ 2049 cpm/seedling in the root vs. 2149 $\pm$ 378 cpm/seedling in the shoot). While the level of labeled protein

Table 2. Effect of nitrate on trifoliin A synthesis and binding to R. trifolii 0403.

	Labeled trifoliin A in root exudate and root surface fractions			
Nitrate level	Root exudate[a]	Percent active[b]	Root surface[a]	Percent active[b]
0	50,550*[c]	100	863*	100
1.5 mM	39,160*	100	3015**	100
15.0 mM	42,660*	2	79***	100

[a] Anti-trifoliin A IgG precipitated protein (cpm/mg root wet wt.).

[b] Percent of immunoprecipitated cpm from each fraction which binds to steamed R. trifolii cells and is blocked by trifoliin A-binding LPS from R. trifolii 0403.

[c] Values in each column with different superscripts are significantly different ($p<0.05$).

immunoprecipitated from the homogenates was low (1-50 cpm/seedling), 85-90% of the labeled lectin was found in the roots of seedlings grown with either nitrate level. The labeled immunoprecipitable protein was about 0.6% of the total labeled protein in the root homogenate of seedlings grown in either 1.5 or 15.0 mM KNO_3, and 0.35-0.45% of the labeled total protein in the shoot homogenate.

The presence of trifoliin A in the root and shoot homogenates was confirmed using western blot analysis with antiserum prepared against seed trifoliin A (Figure 3). The only major protein in the root homogenates which reacted with the diluted antiserum was the 53,000 mol. wt. subunit of trifoliin A (Figure 3). In contrast, the shoot homogenate contained not only the 53,000 mol. wt. lectin subunit, but also several smaller molecular weight polypeptides which reacted strongly with the same diluted antiserum (Figure 3). Based on the relative intensity of the lectin bands on the autoradiogram, there was no obvious effect of nitrate supply on the amount of the 53,000 mol. wt. trifoliin A subunit or the smaller cross-reactive proteins in the shoot homogenate, but there was an apparent reduction in the total amount of trifoliin A per seedling root grown with 1.5 mM nitrate compared with those grown with 15.0 mM nitrate. Controls substituting either preimmune serum or PBS-T (no antiserum) for anti-trifoliin A antiserum during treatment of the blot were negative.

Discussion

Verification of the lectin recognition hypothesis in the Rhizobium-legume symbiosis, and understanding the physiological role of legume lectins in general, requires the examination of the synthesis and fate of lectins in intact infectible and non-infectible seedlings. Lectins have previously been found in the root exudate of different legumes, but the source of the lectin is unknown. The

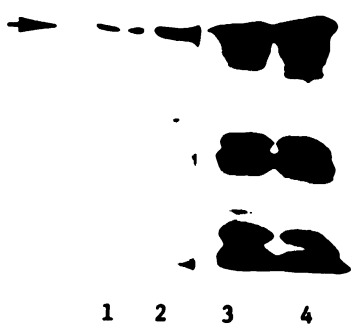

Figure 3. Western blot analysis of the root and shoot homogenates of 2 d-old dark-grown white clover seedlings using anti-trifoliin A antiserum. The root homogenates of seedlings grown with 1.5 mM KNO_3 (lane 1) and with 15.0 mM KNO_3 (lane 2), and the shoot homogenates of seedlings grown with low (lane 3) and high (lane 4) nitrate were detected by autoradiography. The exposure times for lanes 1 and 2 differed from lanes 3 and 4, so the amount of trifoliin A in the root and shoot homogenates should not be directly compared. The arrow points to the 53,000 mol. wt. subunit of trifoliin A.

lectin could be synthesized by the root, transported to the root from the cotyledons, or leaked from the seeds where the lectins are found in abundance. We have shown by the in-vivo incorporation of labeled amino acids that trifoliin A is synthesized in the roots of white clover seedlings and excreted into root exudate. The ability of clover roots to incorporate labeled amino acids into protein, including trifoliin A, diminishes rapidly as the seedling ages. However, this does not imply that older seedlings lack the lectin. The incorporation of labeled amino acids into protein by intact seedlings may only be possible during the period of heterotrophic growth which occurs during the early stages of legume seed germination (Cooper 1977). As the seedlings begin photosynthetic growth and synthesize amino acids, exogenously supplied amino acids may no longer be used although synthesis of the lectin may continue. The lectin synthesis occurring in the root would not appear to be due to "stored" mRNA from the seed which is translated during germination but degraded within 12 h after imbibation (Peumans et al. 1982), because the seedlings used in our experiments were at least 36 h old before labeled amino acids were added.

Some of the labeled immunoprecipitable protein in the root exudate of seedlings grown with low or high nitrate accumulates at the top of the running gel during SDS-PAGE analysis. Separate studies using electron microscopy, SDS-PAGE, and western blot analysis show that this material is aggregated particles which contain many proteins of differing molecular weights, including trifoliin A (Truchet, G.L., Sherwood, J.E., Dazzo, F.B., manuscript in preparation), and which, therefore, immunoprecipitate with anti-trifoliin A IgG.

The addition of KNO_3 affected the amount of labeled lectin in the root surface fraction. Compared to the root surface of seedlings grown under N-free conditions, there is a significant increase in accumulation of newly synthesized trifoliin A when grown with 1.5 mM nitrate and a significant decrease in

accumulation of this lectin when grown with 15 mM nitrate. These results are consistent with immunocytofluorimetric measurements of total trifoliin A on the root surface of 4 d-old seedlings, and could explain the observed effects of nitrate supply on attachment of R. trifolii to clover root hairs (Dazzo and Brill 1978) which was attributed to changes in the number of lectin attachment sites on the root hairs. Similiar effects by nitrate have recently been shown with peas using an enzyme-linked immunoassay (Diaz et al. 1984). In that case, 4 d-old pea seedlings grown in 20 mM nitrate accumulated higher amounts of lectin in the root slime but less lectin was associated with the root cell walls.

Controls in which the plant growth medium was amended with KCl indicated that the changes in lectin distribution and binding-ability were due to nitrate supply and not potassium or the ionic strength of the media. The specific activities of the internal free amino acids available for incorporation into protein were also similar for 2 d-old seedlings grown with different nitrate concentrations, implying that the specific activities of newly synthesized protein in those seedlings would be similar under the different growth conditions. This assumption allows direct comparisons of cpm as a measure of newly synthesized lectin.

The ability of the labeled lectin to interact specifically with heat-killed R. trifolii was quantified to determine if this lectin was active in binding to the bacterial saccharide receptors. Lectin-binding LPS purified from R. trifolii 0403 (Hrabak et al. 1981) was used to inhibit the binding of trifoliin A to these cells to differentiate between specific and non-specific binding to R. trifolii. The amount of labeled immunoprecipitable protein (total trifoliin A) was similar to the level of labeled lectin in the root surface and root exudate fractions which could bind to the bacteria (active trifoliin A) except in the root exudate of seedlings grown with 15.0 mM KNO_3. The lowered apparent Rhizobium-binding

activity of trifoliin A in this root exudate fraction suggests that the active site of the excreted form of the lectin is either modified or masked when synthesized by seedlings in the presence of excess nitrate. This result explains why less trifoliin A was detected by immunofluorescence microscopy in the root exudate of clover seedlings grown with excess nitrate (Dazzo and Hrabak 1981), since this method would only detect active trifoliin A capable of binding to R. trifolii.

Western blot analysis revealed differences in the relative amounts of total trifoliin A in the root and shoot homogenates of 2 day-old seedlings. Shoots had much more total trifoliin A than did the roots, even though little newly synthesized labeled lectin was detected in the shoots. Additionally, the shoots contained numerous smaller molecular weight proteins, apparently degradation products of the lectin which reacted with the anti-lectin antibody. This would be predicted, since lectins have been found in seed protein bodies with storage proteins and proteolytic enzymes which are activated during seed germination (Chrispeels 1984). The shoot lectin and its degradation products are internal, since no trifoliin A is detected on the shoot surface by immunofluorescence microscopy (Dazzo et al. 1978). However, a different process apparently occurs in the seedling roots, where there is trifoliin A containing labeled amino acids, but little detectable degradation of this lectin. This indicates that while seed lectin may act as a food storage protein, the root lectin appears to be excreted to the root surface and the external environment rather than degraded. In addition, nitrate has no obvious affect on the amount or degree of degradation of the shoot lectin, while trifoliin A appears to be more abundant in homogenates of roots grown with high amounts of nitrate. These differences in trifoliin A from the seed and root are consistent with the report that the 120,000 mol. wt. soybean lectin is encoded by 2 genes, one of which is

expressed during seed development and the other expressed during root development (Goldberg et al. 1983).

The infection of legumes roots by homologous rhizobia is known to be regulated in several ways, including the addition of a fixed nitrogen source. Growth of white clover seedlings with high nitrate causes a decrease in the accumulation of newly synthesized, active trifoliin A on the surface of seedling roots and in the root exudate. This represents a mechanism of regulation by nitrate during seedling root development which would affect the interaction between R. trifolii and the clover root.

References

Battieger. B., Newhall, W.J., Jones, R.B. (1982) The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. J. Immunol. Meth. 55, 297-307

Bohlool, B.B., Schmidt, E.L. (1974) Lectins: a possible basis for specificity in the Rhizobium-legume root nodule symbiosis. Science 185, 269-271

Burnette, W.N. (1981) "Western blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112, 195-203

Cooper, C.S. (1977) Growth of the legume seedling. Adv. Agron. 29, 119-139

Chrispeels, M.J. (1984) Biosynthesis, processing and transport of storage proteins and lectins in cotyledons of developing legume seeds. Phil. Trans. R. Soc. Lond. B 304, 309-322

Dazzo, F.B. (1982) Leguminous root nodules. In: Methods in Microbial Ecology, pp. 431-446, Burns, R.G., Slater, J.H., eds. Blackwell Scientific Publications, Oxford

Dazzo, F.B., Brill, W.J. (1978) Regulation by fixed nitrogen of host-symbiont recognition in the Rhizobium-clover symbiosis. Plant Physiol. 62, 18-21

Dazzo, F.B., Brill, W.J. (1979) Bacterial polysaccharide which binds Rhizobium trifolii to clover root hairs. J. Bacteriol. 137, 1362-1373

Dazzo, F.B., Hrabak, E.M. (1981) Presence of trifoliin A, a Rhizobium-binding lectin, in clover root exudate. J. Supramolec. Struct. Cell. Biochem. 16, 133-138

Dazzo, F.B., Hrabak, E.M. (1982) Lack of a direct nitrate-trifoliin A interaction in the Rhizobium-clover symbiosis. Plant Soil 69, 259-264.

Dazzo, F.B., Hubbell, D.H. (1975) Cross-reactive antigens and lectin as determinants of symbiotic specificity in the Rhizobium-clover association. Appl. Microbiol. 30, 1017-1033

Dazzo, F.B., Yanke, W.E., Brill, W.J. (1978) Trifoliin: a Rhizobium recognition protein from white clover. Biochim. Biophys. Acta 539, 276-286

Diaz, C.L., Lems-van Kan, P., Van der Schaal, L.A.M., Kijne, J.W. (1984) Determination of pea (Pisum sativum L.) root lectin using an enzyme-linked immunoassay. Planta: 161 (in press).

Gade, W., Schmidt, E.L., Wold, F. (1983) Evidence for the existence of an intracellular root lectin in soybeans. Planta 158,108-110.

Gietl, C., Ziegler, H. (1979) Lectins in the excretion of intact roots. Naturwissenshaften 66,161-162.

Goldberg, R.B., Hoschek, G., Vodkin, L.O. (1983) An insertion sequence blocks the expression of a soybean lectin gene. Cell 33, 465-475

Goldstein, LJ., Hughes, R.C., Monsigny, M., Osawa, T., Sharon, N. (1980) What should be called a lectin? Nature (London) 285, 66

Hrabak, E.M., Urbano, M.R., Dazzo, F.B. (1981) Growth-phase dependent immunodeterminants of Rhizobium trifolii lipopolysaccharide which binds trifoliin A, a white clover lectin. J. Bacteriol. 148, 697-711

Irie, S., Sezaki, M., Kato, Y. (1982) A faithful double stain of proteins in polyacrylamide gels with Coomassie blue and silver. Anal. Biochem. 126, 350-354

Key, J.L., Lin, C.Y., Chen, Y.M. (1981) Heat shock proteins of higher plants. Proc. Natl. Acad. Sci. USA 78,3526-3530

Kijne, J.W., van der Schaal, L.A.M., de Vries, G.E. (1979) Pea lectins and the recognition of Rhizobium leguminosarum. Plant Sci. Lett. 18, 65-74

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227, 680-685

Munns, D.N. (1968) Nodulation of Medicago sativa in solution culture. III. Effects of nitrate on root hairs and infection. Plant and Soil 29, 33-49

Nutman, P.S. (1962). The relation between root hair infection by Rhizobium and nodulation in Trifolium and Vicia. Proc. Roy. Soc. B (London) 156,122-137

Peumans, W.J., Stinissen, H.M., Carlier, A.R. (1982) Lectin synthesis in developing wheat and rye embryos. Planta 156,41-44

Rosen, H. (1957) A modified ninhydrin colorimetric analysis for amino acids. Arch. Biochem. Biophys. 67, 10-15

Sengupta-Gopalan, C., Pitas, J.W., Hall, T.C. (1984) Re-examination of the role of lectin in Rhizobium-Glycine symbiosis. In: Advances in nitrogen fixation research, p. 427. Veeger, C., Newton, W.E., eds. Nijhoff/ Junk Publishers, the Hague.

Sherwood, J.E., Vasse, J.M., Dazzo, F.B., Truchet, G.L. (1984) Development and trifoliin A-binding ability of the capsule of Rhizobium trifolii. J. Bacteriol. 159, 145-152

Witte, O.N., Wirth, D.E. (1979) Structure of the murine leukemia virus envelope glycoprotein precursor. J. Virol. 29, 735-743

CHAPTER THREE

DEVELOPMENT AND TRIFOLIIN A-BINDING ABILITY OF THE CAPSULE OF *RHIZOBIUM TRIFOLII* 0403

John E. Sherwood, Jacques M. Vasse, Frank B. Dazzo and Georges L. Truchet

Abstract

The age-dependent lectin-binding ability of *Rhizobium trifolii* 0403 capsular polysaccharide (CPS) has been examined by following the development of the capsule and its ability to interact with the white clover lectin, trifoliin A. Bacteria grown on agar plates for 3, 5, 7, 14, and 21 days were examined by electron microscopy and immunofluorescence microscopy with antibodies prepared against either *R. trifolii* 0403 CPS or trifoliin A after pretreatment with the lectin. The capsule began to develop at one pole by day 3 and completely surrounded the cells in cultures incubated for 5 days or longer. The capsular polysaccharide on cells cultured for 3 and 5 days was completely reactive with trifoliin A, became noticeably less reactive by day 7, and only reactive with the lectin at one pole of a few cells after that time. The quantity and location of lectin receptors on bacteria of different ages directly correlated with their attachment in short-term clover root hair-binding studies. Cells from 3 or 21 day-old cultures attached almost exclusively in a polar fashion, while cells grown for 5 days attached to root hairs randomly and in the highest numbers. CPS isolated from a 5 day-old culture had higher lectin-binding

ability than CPS from 3 and 7 day-old cultures, while the CPS from a 14 day-old culture had the lowest. Chemical analyses of the isolated CPS showed changes in the levels of uronic acids (as glucuronic acid), pyruvate, and O-acetyl substitutions with culture age, but the neutral sugar composition remained relatively constant. These results provide evidence that the age-dependent distribution of lectin receptors dictates the level and orientation of attachment of R. trifolii 0403 to clover root hairs.

Introduction

The capsular polysaccharide surrounding bacteria is important for various cellular functions in nature, including adhesion to solid surfaces, pathogenicity, and protection from predation, desiccation, and other environmental stresses (9, 19). The development of bacterial capsules has been examined microscopically using temperature-sensitive mutants of Escherichia coli (2) and Haemophilis influenzae transformants of mutants lacking capsules (7). E. coli appears to have 10-40 random sites of capsular excretion around the cell. The majority of newly encapsulated H. influenza cells show thin capsules completely surrounding the cells, although some capsules appear to develop polarly. Bacteria of the genus Rhizobium, which form a nitrogen-fixing symbiosis with leguminous plants, differ in terms of encapsulation (18). Slow-growing R. japonicum strains typically exhibit a polar capsule (29, J. Vasse et al., in preparation), while R. trifolii, R. leguminosarum, and R. phaseoli form a complete capsule (15, 18). R. meliloti cells typically lack a capsule (18, 24), although polar fibrils can generally be seen (24). The percentage of encapsulated cells of R. trifolii, and the amount of polysaccharide synthesized, varies with the strain and cultural conditions (18). The development of the rhizobial capsule is relevent to the symbiosis due to the specific interaction between the capsular polysaccharide and lectins synthesized

by the legume host (4, 6, 13). It has been hypothesized that the specific attachment of rhizobia to host root hairs, an early event in the infection process, results from the interaction between the capsular polysaccharide (CPS) and lectin on the host root (13, 22, 25, 27).

A phenomenon which has been noted for several Rhizobium species is the age-dependent nature of lectin-binding. R. japonicum 3I1b138 loses its ability to interact with the soybean lectin after mid-exponential growth, apparently due to the methylation of galactosyl residues in the polysaccharide and a reduction in encapsulation (23). Transient lectin-binding ability also occurs with certain strains of R. leguminosarum, which interact with the pea lectin optimally when the bacteria are in early exponential phase (30). R. trifolii 0403, grown either in broth or on agar plates, shows a transient ability to bind the clover lectin, trifoliin A (15, 21). The lipopolysaccharide (LPS) of R. trifolii 0403 grown in broth culture has growth phase-dependent increases in the level of quinovosamine, which occurs at the same time (early stationary phase) that the LPS becomes optimally reactive with trifoliin A (21). While it has been shown that an acidic polysaccharide from the capsule of R. trifolii 0403 binds to trifoliin A (11), the transient ability of the isolated CPS to bind trifoliin A was not examined. Cadmus et al. (5) found age-dependent variations in the levels of pyruvate and O-acetyl substitutions in several fast-growing species Rhizobium, including R. trifolii, although lectin binding was not investigated.

In order to characterize the age-dependent nature of the binding of the capsule of R. trifolii to trifoliin A, we have examined cells of R. trifolii 0403 for i) the development of the capsule, ii) the ability of the capsule at various stages of development to interact with the lectin, and iii) the effect of culture age on the binding of the cells to clover root hairs.

Materials and methods

Bacterial cultures. Rhizobium trifolii 0403 was obtained from the Rothamsted Experimental Station (Harpenden, England). Cultures were maintained on BIII agar medium (10) and grown at 30°C. Broth cultures were grown in BIII medium as previously described (21). All transfers were made with a 5 day-old plate culture as inoculum.

Immunofluorescence microscopy. R. trifolii 0403 of various ages was suspended from BIII plates in phosphate buffered saline (PBS) (16), and centrifuged at 3,000 x g for 20 min at 4°C. The encapsulated cells in the upper, soft layer of the pellet were resuspended and washed twice in PBS under the same conditions. The soft pellet was resuspended in PBS, heat-fixed to microscope slides, rinsed with distilled water and air-dried. Antiserum prepared in rabbits against whole, encapsulated cells of R. trifolii 0403 was adsorbed exhaustively with steamed, washed, broth-grown bacteria in mid-exponential and early stationary growth phases. This adsorbed antiserum no longer reacted with unencapsulated cells. Trifoliin A and rabbit antibody against this clover lectin were prepared as previously described (16). The cells were labeled for indirect immunofluorescence with FITC goat anti-rabbit IgG (Miles-Yeda, Ltd., Israel).

Electron microscopy. Bacteria for electron microscopy were prepared as described above. The cells were contrasted by the glutaraldehyde/ruthenium red/uranyl acetate (G/RR/UA) procedure (24). In some cases, bacteria were pretreated for 30 min at room temperature with immunoaffinity-purified trifoliin A (12) coupled to colloidal gold (20) prior to treatment by the G/RR/UA method. Specimens were observed on a Phillips 300 transmission electron microscope. Cells were also treated by freeze drying and shadowing (24). The bacteria were treated by G/RR/UA, frozen in liquid nitrogen, sublimed in a vacuum (1×10^{-7} torr) for 10 min each at -120°C, -100°C, and -80°C. The

specimens were shadowed by platinum-carbon evaporation at -150°C at a 45° angle and examined with a Hitachi 600 electron microscope at 75 kV.

Root hair binding assay. A suspension of washed, encapsulated R. trifolii 0403 was adjusted to concentrations of either 1 x 10^6 or 1 x 10^8/ml in Fahraeus (N-free) medium (10). Surface-sterilized seeds of Trifolium repens var. Louisiana Nolin were germinated into humid air for 48 h. The seedlings were incubated with 5 ml of the bacterial suspension in polypropylene beakers for 1 h at room temperature with gentle circular shaking. The seedlings were gently washed in sterile Fahraeus medium, mounted on a microscope slide and observed by phase contrast microscopy. Bacteria attached to root hairs 200 µm long were counted (10), noting both the total number of attached cells per root hair and the orientation of attachment.

Isolation of capsular polysaccharide. Lawns of R. trifolii 0403 were prepared using an inoculum from a 5 day-old culture of cells which had their capsules removed by vigorous agitation in sterile PBS, centrifugation at 12,000 x g, and two washes in PBS. The bacteria were grown at 30°C for 3, 5, 7, 14, or 21 d and prepared as described above (Immunofluorescence microscopy section). The encapsulated cells were rapidly stirred at room temperature with 0.5 M NaCl in PBS for 1 h to extract the capsular polysaccharide. The cells were removed by centrifugation at 10,000 x g at room temperature until the supernatant was clear. The extracted polysaccharide was precipitated in 3 volumes of cold ethanol, redissolved and dialyzed against deionized, distilled water at 4-10°C, and lyophilized.

Agglutination inhibition assay. Bacterial agglutination inhibition assays were performed as described previously (15) to measure the level of binding of CPS to trifoliin A. R. trifolii 0403 cells used for the assay were grown on BIII agar at 30°C for 5 days. Purified trifoliin A was adjusted to a concentration of 260

µg/ml as measured by the Bio-Rad Protein Assay (Richmond, Ca.). Solutions of the isolated polysaccharides (200 µg glucose equivalents/ml) in M buffer (16) were preincubated with equal volumes of the lectin solution for 1 h at 30°C. Serial dilutions were then made in M buffer, bacteria were added (200 µl of a suspension containing 1.2 x 10^9 cells/ml) and then incubated at 30°C for an additional 4 h. The degree of inhibition is expressed as agglutination inhibition units (decrease in titer/100 µg glucose equivalent) (21).

Carbohydrate analysis. The isolated polysaccharides were examined for neutral sugars by the phenol-sulfuric acid assay (17), for uronic acids by the method of Blumenkrantz and Asboe-Hansen (3), and for pyruvate and O-acetyl substitutions as described by Sutherland (28). The level of pyruvylation was also measured by high performance liquid chromatography (HPLC) after hydrolysis of the pyruvate groups in 0.005 N H_2SO_4 at 100°C for 3 h. The acid-treated polysaccharide was precipitated in 3 volumes of ethanol containing 0.1 mM $MgSO_4$. After centrifugation at 1000 x g for 5 min, the ethanol in the supernatant was evaporated under nitrogen. Pyruvate in the remaining solution was quantitated on a Water's HPLC using a Bio-Rad HPX-87H Organic Acid Analysis column at 50°C, 0.005 N H_2SO_4 as the solvent, and detected at 210 nm with a Gilson Holochrome UV Monitor. The sugar composition of the CPS was determined by analyzing trimethylsilyl derivatives prepared with Tri-Sil Z (Pierce Chemical Co., Rockford, Il.) following methanolysis overnight at 80°C in 1 N HCl in methanol (8). The derivatized sugars were separated on 3% SE-30 on Chromosorb W(HP) (Pierce Chemical Co.) at 150°C using a Varian 3740 Gas Chromatograph. Peak areas were quantitated on a Hewlett-Packard 3390A Integrator and corrected for detector response by comparison with derivatized standard sugars.

Results

The development of the capsule of *R. trifolii* 0403 was found to be directly related to the conditions of growth. In shaken broth cultures, no encapsulation was detected by the G/RR/UA method, regardless of the culture age. Fibrils were produced at one cell pole in early exponential phase (Figure 1a) and emitted into the growth medium, forming a fibrillar net (Figure 1b). Encapsulated cells were ocassionally observed (<1%) in broth culture at early stationary phase when examined by immunofluorescence (figure not shown).

In contrast, *R. trifolii* 0403 frequently formed capsules when cultivated on agar plates (Table 1). Immunofluorescence microscopy using anti-capsule antibody showed that the capsule began to develop at one pole by 3 days (Figure 2a), and completely surrounded the majority of cells by day 5 (Figure 2b). Bacteria remained completely encapsulated throughout the remaining 16 days of incubation (Figure 2c, d and e), although the size of the capsule and the intensity of immunofluorescence staining of the capsular antigen varied (Figure 2c). The percentage of encapsulated cells remained constant in 5-14 day-old cultures, but decreased in the 21 day-old culture (Table 1).

Examination of cells by electron microscopy confirmed that the early development of the capsule was polar (Figure 3a and b), and completely surrounded most cells in 5 day-old cultures (Figure 3c). The capsule remained intact through the remainder of the incubation period as shown by freeze drying (Figure 4) and the G/RR/UA method (figure not shown).

The ability of encapsulated cells to bind to the clover lectin, trifoliin A, changed with culture age (Table 1). Indirect immunofluorescence showed that lectin bound to few cells grown for 3 days, and, in those cases, only to one pole (Figure 5a). By 5 days, lectin receptors were distributed evenly in the fully developed capsule (Figure 5b). The intensity and eveness of fluorescence

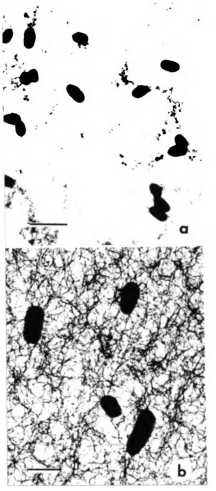

Figure 1. R. trifolii 0403 grown in shaken broth culture. Cells from a) early exponential and b) stationary phases were examined by electron microscopy. Bars, 2 μm.

Table 1. Effect of culture age on the encapsulation and lectin-binding ability of *R. trifolii* 0403 grown on BIII agar.[a]

Culture age (days)	% Encapsulation		Distribution of capsular trifoliin A receptors	
	Full	Partial	% Uniform	% Nonuniform
3	0	23	1	16
5	72	16	76	12
7	76	12	70	21
14	76	11	4	14
21	42	12	5	20

[a]Expressed as a percentage of the total population examined by immunofluorescence with anti-capsule or trifoliin A and anti-trifoliin A antibodies.

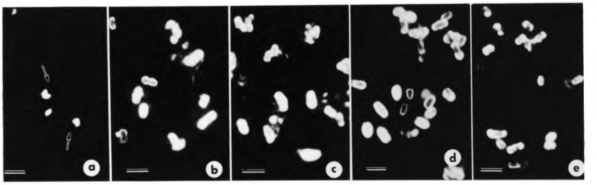

Figure 2. Encapsulation of R. trifolii 0403 detected by indirect immunofluorescence using homologous anti-CPS antiserum. Bacteria were grown for a) 3 days, b) 5 days, c) 7 days, d) 14 days, and e) 21 days. Arrows indicate location of cells when viewed by phase contrast microscopy. Bars, 2 µm.

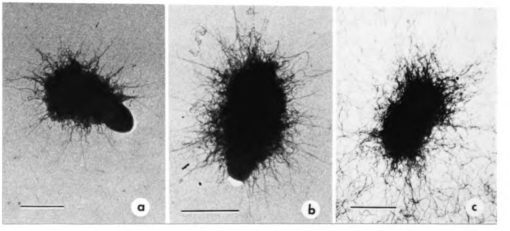

Figure 3. Development of the capsule of R. trifolii 0403 examined by transmission electron microscopy. Bacteria are from 4 day (a and b) or 5 day (c) cultures. Bars, 2 µm.

decreased by day 7 (Figure 5c), although the capsule retained the ability to bind trifoliin A at that time. The ability of encapsulated cells to bind trifoliin A decreased in intensity and percentage of reactive cells by days 14 and 21, when lectin receptors were predominately detected at one pole (Figure 5d, Table 1).

These immunofluorescence studies were confirmed by electron microscopy using trifoliin A coupled to electron-dense colloidal gold to locate lectin receptors. The newly developed polar capsule on cells in 3 day-old cultures uniformly bound trifoliin A (Figure 6a), as did the complete capsule on cells from 5 day-old cultures (Figure 6b). In contrast, fully encapsulated cells cultured for 14 and 21 days generally bound the lectin more at one pole (Figure 6c). The capsules of cells cultured for 21 days and treated by the G/RR/UA method were much less electron-dense than cells from younger cultures (compare Figure 6a, b, and c), and other extracellular fibrillar structures which stained negatively were also evident (Figure 6c).

The relationship between these developmental changes of the capsule and the attachment of R. trifolii 0403 to clover root hairs was examined after 1 h incubation of the bacteria at two different inoculum sizes with clover seedlings. When a 3 day-old culture was used at the higher inoculum (1×10^8 bacteria/ml), the bacteria attached almost exclusively in a polar orientation (Figure 7a), matching the polar location of the lectin-binding capsule (Figures 2a, 3a, 5a, and 6a). Similarly, R. trifolii 0403 from 5 day-old cultures, which have lectin receptors uniformly surrounding the fully encapsulated bacteria, attached to root hairs in a random orientation and clumped extensively at the root hair tips (Figure 7b). The few cells from cultures grown for 14 or 21 days which bound to root hairs did so polarly (Figure 7c). These results were confirmed by direct microscopic counts of attached bacteria and their orientation of attachment using a lower inoculum of 1×10^6 cells/ml (Figure 8). Bacterial attachment to

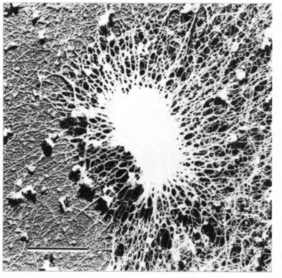

Figure 4. R. trifolii 0403 from 21 day-old cultures examined by transmission electron microscopy after freeze-drying and shadowing. Bar, 1 µm.

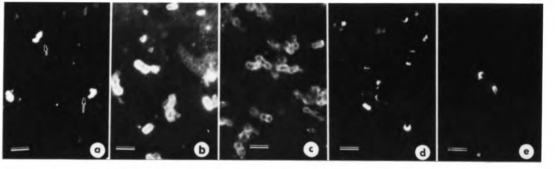

Figure 5. Binding of trifoliin A to the capsule of R. trifolii 0403 as shown by indirect immunofluorescence. Bacteria were grown for a) 3 days, b) 5 days, c) 7 days, d) 14 days, and e) 21 days. Arrows indicate position of cells when viewed by phase contrast microscopy. Bars, 2 µm.

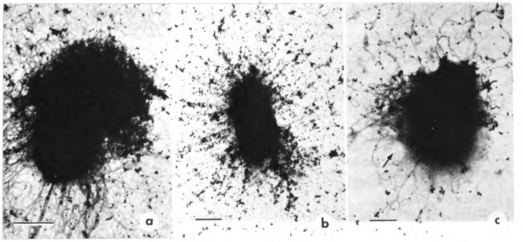

Figure 6. Localization of trifoliin A-binding sites on the capsule of R. trifolii 0403 by transmission electron microscopy. Bacteria were grown for a) 3 days, b) 5 days, or c) 21 days and reacted with trifoliin A coupled with colloidal gold. Note the negatively stained fibrillar material in figure 6c (arrow). Bars, 0.5 μm.

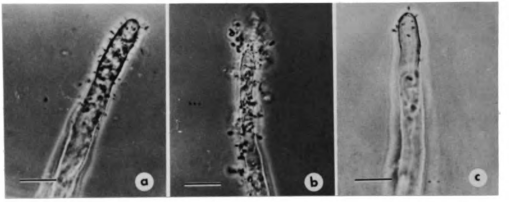

Figure 7. Attachment of R. trifolii 0403 to clover root hairs. Bacteria were grown for a) 3 days, b) 5 days, and c) 21 days and incubated with clover seedlings for 1 h. Bars, 20 μm.

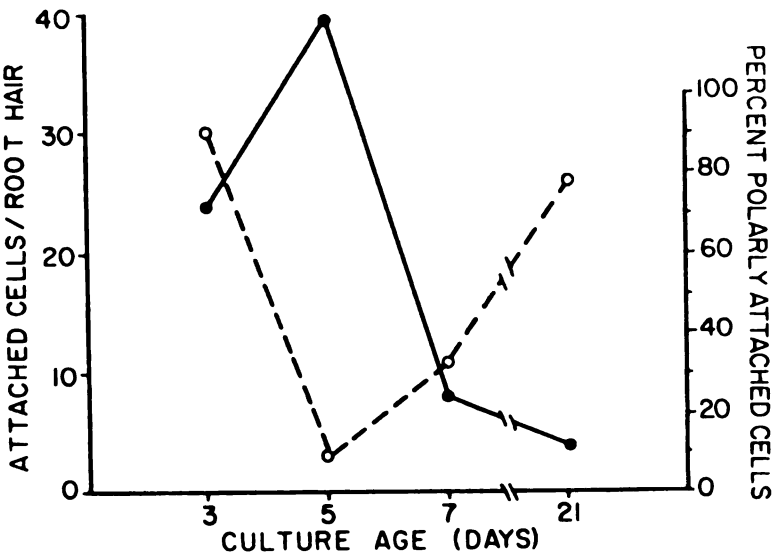

Figure 8. Effect of culture age on the quantitative attachment of R. trifolii to clover root hairs. Numbers of bacteria attached (●) and orientation of attachment (o) are shown.

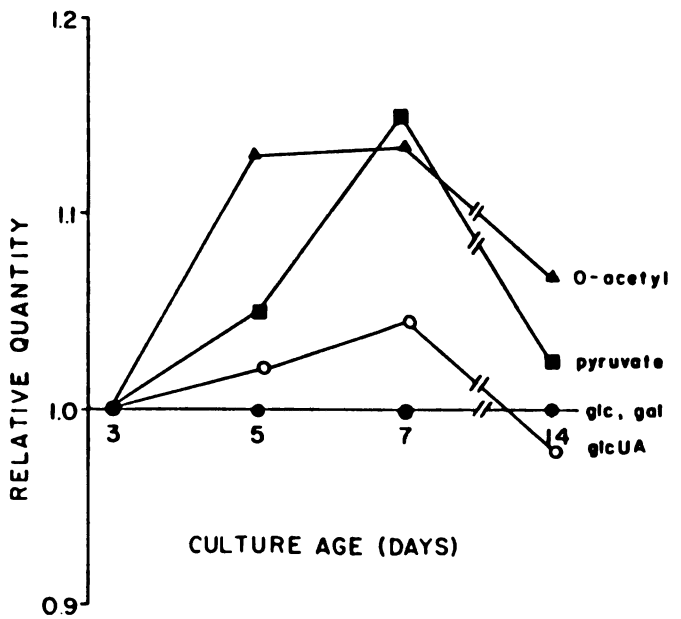

Figure 9. Relative quantities of the components of the capsular polysaccharide of R. trifolii 0403 at different culture ages. All values are standardized to the levels found in the CPS from 3 day-old cultures.

Table 2. Effect of culture age on the interaction between isolated CPS and trifoliin A.

Culture age (days)[a]	Bacterial agglutination inhibition[b]
3	502
5	852
7	686
14	0

[a]CPS isolated from plate cultures at different ages and adjusted to 100 µg glucose equivalents per ml.

[b]Inhibitory units per mg of glucose equivalents; trifoliin A specific activity was 985 units per mg protein.

root hairs was greatest in number with the least amount of polar attachment with cells from 5 day-old cultures. When receptors for trifoliin A were more abundant at one cell pole (i.e., in 3 and 21 day-old cultures), attachment was almost exclusively polar. Although this also coincided with the location of the developing capsule of young cells, such was not the case with bacteria from 14 and 21 day-old cultures, which were most often fully encapsulated (Table 1).

The capsular polysaccharide was extracted from plate cultures of R. trifolii 0403 at different culture ages and examined for the ability to bind purified trifoliin A by bacterial agglutination inhibition (Table 2). Lectin-binding activity of the isolated CPS was highest from 5 day-old cultures, intermediate from 3 and 7 day-old cultures, and least from cells grown for 14 days.

Changes were found in both the glycosyl and non-glycosyl composition of the capsular polysaccharides isolated from cultures at different ages (Figure 9). The relative level of neutral hexoses remained constant (60% of the total weight) throughout the incubation period, as did the the ratio of glucose to galactose (5:1), measured as their trimethylsilyl derivatives. Both the colorimetric assay and gas chromatographic analysis detected a slight increase in the relative level of glucuronic acid in the CPS of cells grown for 5 and 7 days. More striking, however, were the transient increases in the non-glycosyl substitutions. The level of pyruvate substitutions increased from 9.7 % (w/w) in CPS from 3 day-old cultures to a maximum of 10.8% (w/w) in 7 day-old cultures. This increase was detected by the colorimetric assay (Figure 9) and confirmed by HPLC analysis. The level of O-acetylation rose from 7.5% (w/w) in the CPS from 3 day-old cultures to 8.3% (w/w) in the CPS from 5 and 7 day-old cultures. The levels of both pyruvate and O-acetyl substitutions decreased slightly in the CPS from a 14 day-old culture, although not to the level found with very young (3 day) cultures.

Discussion

The development of the capsule and localization of the lectin receptors in the capsule of R. trifolii 0403 were examined using immunofluorescence and immunoelectron microscopy. There is a fundamental difference between the relationship of encapsulation and ability to bind trifoliin A, based on whether the cells are grown in shaken broth culture or on agar surfaces. In shaken broth culture, these bacteria rarely form complete capsules, although >90% of the cells uniformly bind trifoliin A at a specific time in early stationary phase (21). The interaction between the lectin and cells in shaken broth culture can therefore be attributed primarily to the lectin-binding LPS (21). In contrast, cells grown on agar surfaces form capsules which also bind trifoliin A at a specific culture age (15). Therefore, in order to study the development of the lectin-binding capsule of R. trifolii 0403, we used cultures grown on solid medium. In addition, growth on an agar surface may more closely approximate growth in the natural environment than does growth in shaken broth culture (9). Our studies confirm the age-dependent ability of encapsulated cells of R. trifolii 0403 to bind trifoliin A, and that encapsulated cells cultivated for 5 days on BIII agar react most strongly with trifoliin A (15). In addition, a direct correlation between the distribution of the lectin receptors within the capsule and the orientation of attachment of R. trifolii 0403 to clover root hairs was demonstrated. The direct interaction between isolated CPS and purified lectin was quantitated and also found to change with culture age.

The capsule of R. trifolii 0403 grown on BIII agar would be described, according to the scheme of Costerton et al. (9), as rigid, since it can exclude India ink particles (unpublished observation), and peripheral, since it remains intact under certain conditions but may be shed directly into the medium under other conditions. The CPS forms a discrete layer bound to the bacterial surface

and is distinct from EPS, which is not bound to the cell. Thus, the CPS of plate-grown cells can be physically distinguished and separated from EPS.

Immunofluorescence and electron microscopy indicate that the capsule of R. trifolii 0403 is first excreted from the cell at one pole. This pattern of capsule development differs from that in E. coli, in which the polysaccharide is excreted from many places around the cell (2). Synthesis of the capsule by R. trifolii could remain polar with the capsule expanding from one pole to "engulf" the cell, or, alternatively, the points of synthesis and excretion of capsular polysaccharide from the fluid outer membrane may redistribute more evenly around the cell as the culture ages. Evidence suggesting that the first possibility is more likely includes: i) the acidic fibrils of cells grown in shaken broth culture remain polar during growth, ii) the capsules of cells in young, plate-grown cultures are polar, and iii) root exudate enzymes partially degrade the capsule of living R. trifolii 0403 cells leaving a polar capsule, but completely degrade the capsule of killed cells (14). The lack of capsules on 7 day-old plate cultures of R. trifolii 0403 reported previously (15) was probably due to the centrifugation steps used to prepare the cells for electron microscopy. The more gentle methods used in this study clearly show that the cells remain fully encapsulated after 5 days of growth.

The ability of the capsule of R. trifolii 0403 to bind to trifoliin A is maximal on cells grown for approximately 5 days. The binding of trifoliin A to encapsulated cells grown for 7 days is weaker and uneven, while lectin receptors are generally polar after that time. The orientation of bacterial attachment to root hairs reflects the location of lectin receptors rather than the presence of the capsule itself. Cells with polar lectin receptors, therefore, bind to root hairs polarly, regardless of whether they have a polar or uniform capsule, and cells with a uniform distribution of lectin receptors in the capsule

bind to root hairs randomly. The quantity of cells which attach reflects the relative proportion of cells in the inoculum which can bind trifoliin A. Thus, 5 day-old cultures, which contain the highest percentage of cells which uniformly bind trifoliin A, attach to clover root hairs in the highest numbers and in a random orientation. The bacterial clumping at the root hair tip may be mediated by trifoliin A, as it is Rhizobium-specific and inhibited by 2-deoxy-D-glucose (which blocks the interaction between trifoliin A and its saccharide receptors), at inoculum sizes below 1×10^9 bacteria/seedling (Dazzo, F.B., et al., manuscript in preparation). While cells from 14 and 21 day-old cultures have uniform capsules, they bind to root hairs polarly which rules out a non-specific adherence of the capsule itself. The polar attachment of cells from 3 and 21 day-old cultures to clover root hairs occurs within 1 h, and therefore does not involve rhizosphere enzymes which require at least 4 h to convert a full capsule into a polar capsule (14).

A relatively crude preparation of isolated CPS was used for lectin-binding assays and chemical analysis to minimize the possibility of removing any capsular fractions which might interact with the lectin, although ethanol precipitation and dialysis may have resulted in the loss of low molecular weight compounds which affect root hair infection (1). The CPS isolated from cells at all culture ages contained less than 3% (w/w) neutral polysaccharides which are soluble in 2% cetylpyridinium chloride (unpublished data) and lacked LPS-specific sugars of R. trifolii 0403 (21) which would have been detected by gas chromatography. Agglutination inhibition studies showed that the degree of lectin-binding by the isolated CPS parallels the age-dependent ability of R. trifolii 0403 cells to react with trifoliin A (15), and is optimal in both cases with 5 day-old cultures. Changes in the CPS, therefore, can explain the age-dependent interaction of plate-grown cells with the lectin. Identification of

these changes should provide clues to explain the chemical basis for binding of CPS to trifoliin A. The glycosyl composition is consistent with that reported for the EPS of broth-grown R. trifolii 0403 isolated during stationary phase (26), in which the repeating oligosaccharide subunit contains 5 glucose, 1 galactose, and 2 glucuronic acid moieties. Although the neutral sugar composition of the CPS remained unchanged during growth, slight changes were found in uronic acid levels using procedures reported to optimize the recovery of uronic acids from polysaccharides (8). These changes may be due to non-glycosyl substitutions which affect their detection and quantitation, or to synthesis of CPS with less uronic acid per repeating unit. Pyruvate and O-acetyl substitutions increase during growth to a greater degree than do the uronic acids. While similar increases have been shown for the EPS of broth grown R. trifolii L-158 (5), the degree of substitution begins to decrease in the CPS after 7 days of growth on agar plates. The excretion of less substituted polysaccharides could explain the reduction in the percentage of these substitutions in the EPS of R. meliloti L-89 reported by Cadmus et al. (5). The age-dependent changes in levels of pyruvate and O-acetyl substitutions occurred in parallel with the ability of the isolated CPS to bind trifoliin A. Separate studies using one and two-dimensional ^{1}H-NMR analysis of oligosaccharide fragments of the CPS of R. trifolii 0403 confirm that the non-carbohydrate substitutions change with culture age and that these changes affect the affinity of the oligasaccharides with trifoliin A (Abe et al., manuscript in preparation).

The bacterial capsule appears to be a dynamic structure surrounding the cell rather than a simple homogeneous entity. In pure culture, the ability of encapsulated cells of Rhizobium to interact with the lectins of their host plants changes with culture age (15, 22, 23, 30). In addition, localized alterations in the capsule of R. trifolii which affect its affinity for trifoliin A occur when the

bacterium is inoculated into the clover root environment (14). These modifications have a profound impact on the ability of R. trifolii to attach to clover root hairs, an early step in the formation of the nitrogen-fixing symbiosis.

List of references

1. Abe, M., A. Amemura, and S. Higashi. 1982. Studies on cyclic ß-1,2-glucan obtained from periplasmic space of Rhizobium trifolii cells. Plant Soil 64:315-324.
2. Bayer, M.E., and H. Thurow. 1977. Polysaccharide capsule of Escherichia coli: microscope study of its size, structure, and sites of synthesis. J. Bacteriol. 130:911-936.
3. Blumenkrantz, N., and G. Asboe-Hansen. 1973. New method for quantitative determination of uronic acids. Anal. Chem. 54:484-489.
4. Bohlool, B.B., and E.L. Schmidt. 1974. Lectins: a possible basis for specificity in the Rhizobium-legume root nodule symbiosis. Science 185:269-271.
5. Cadmus, M.C., K.A. Burton, and M.E. Slodki. 1982. Growth-related substituent changes in the exopolysaccharides of fast-growing rhizobia. Appl. Environ. Microbiol. 44:242-245.
6. Calvert, H.E., M. Lalonde, T.V. Bhuvaneswari, and W.D. Bauer. 1978. Role of lectins in plant-microorganism interactions 1V. Ultrastructural localization of soybean lectin binding sites on Rhizobium japonicum. Can. J. Microbiol. 24:785-793.
7. Catlin, B.W., and V.R. Tartagni. 1969. Delayed multiplication of newly synthesized capsulated transformants of Haemophilus influenzae detected by immunofluorescence. J. Gen. Microbiol. 56:387-401.
8. Clamp, J.R., T. Bhatti, and R.E. Chambers. 1971. The determination of carbohydrate in biological materials by gas-liquid chromatography. p. 229-344. In D. Glick (ed.), Methods of Biochemical Analysis, Vol. 19. John Wiley and Sons, Inc., N.Y.
9. Costerton, J.W., R.T. Irvin, and K.J. Cheng. 1981. The bacterial glycocalyx in nature and disease. Ann. Rev. Microbiol. 35:299-324.
10. Dazzo, F.B. 1982. Leguminous root nodules. p. 431-446. In R.G. Burns and J.H. Slater (ed.), Experimental Microbial Ecology. Blackwell Scientific Publications, Oxford.
11. Dazzo, F.B., and W.J. Brill. 1979. Bacterial polysaccharide which binds Rhizobium trifolii to clover root hairs. J. Bacteriol. 137:1362-1373.
12. Dazzo, F.B., and E.M. Hrabak. 1981. Presence of trifoliin A, a Rhizobium-binding lectin, in clover root exudate. J. Supramol. Struct. Cell. Biochem. 16:133-138.
13. Dazzo, F.B., and D.H. Hubbell. 1975. Cross-reactive antigens and lectin as determinants of symbiotic specificity in the Rhizobium-clover association. Appl. Microbiol. 30:1017-1033.
14. Dazzo, F.B., G.L. Truchet, J.E. Sherwood, E.M. Hrabak, and A.E. Gardiol. 1982. Alteration of the trifoliin A-binding capsule of Rhizobium trifolii 0403 by enzymes released from clover roots. Appl. Environ. Microbiol. 44:478-490.

15. Dazzo, F.B., M.R. Urbano, and W.J. Brill. 1979. Transient appearance of lectin receptors on Rhizobium trifolii. Curr. Microbiol. 2:15-20.
16. Dazzo, F.B., W.E. Yanke, and W.J. Brill. 1978. Trifoliin: a Rhizobium recognition protein from white clover. Biochim. Biophys. Acta 539:276-286.
17. Dubois, M.K., K.A. Gillies, J.K. Hamilton, P.A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350-356.
18. Dudman, W.F. 1968. Capsulation in Rhizobium species. J. Bacteriol. 95: 1200-1201.
19. Dudman, W.F., 1977. The role of surface polysaccharides in natural environments. p. 357-414. In L.W. Sutherland (ed.), Surface Carbohydrates of the Procaryotic Cell. Academic Press, N.Y.
20. Horisberger, M., J. Rosset, and H. Bauer. 1975. Colloidal gold granules as markers for cell surface receptors in the scanning electron microscope. Experientia 31:1147-1151.
21. Hrabak, E.M., M.R. Urbano, and F.B. Dazzo. 1981. Growth-phase dependent immunodeterminants of Rhizobium trifolii lipopolysaccharide which binds trifoliin A, a white clover lectin. J. Bacteriol. 148:697-711.
22. Kato, G., Y. Maruyama, and M. Nakamura. 1981. Involvement of lectins in Rhizobium-pea recognition. Plant Cell Physiol. 66:609-614.
23. Mort, A.J., and W.D. Bauer. 1980. Composition of the capsular and extracellular polysaccharides of Rhizobium japonicum. Plant Physiol. 66:158-163.
24. Mutaftschiev, S., J. Vasse, and G. Truchet. 1982. Exostructures of Rhizobium meliloti. FEMS Microbiol. Lett. 13:171-175.
25. Paau, A.S., W.T. Leps, and W.J. Brill. 1981. Agglutinin from alfalfa necessary for binding and nodulation by Rhizobium meliloti. Science 213:1513-1515.
26. Robertson, B.K., P. Aman, A.G. Darvill, M. McNeil, and P. Albersheim. 1981. Host-symbiont interactions. V. The structure of acidic polysaccharides secreted by Rhizobium leguminosarum and Rhizobium trifolii. Plant Physiol. 67:389-400.
27. Stacey, G., A.S. Paau, and W.J. Brill. 1980. Host recognition in the Rhizobium-soybean symbiosis. Plant Physiol. 66:609-614.
28. Sutherland, L.W. 1969. Structural studies on colonic acid, the common exopolysaccharide found in Enterobacteriaceae, by partial hydrolysis. Biochem. J. 115:935-945.
29. Tsien, H.C., and E.L. Schmidt. 1977. Polarity in the exponential-phase Rhizobium japonicum cell. Can. J. Microbiol. 23:1274-1284.
30. van der Schaal, L, J. Kijne, C. Diaz, and F. van Iren. 1983. Pea lectin binding by Rhizobium, p. 531-538. In T.C. Bog-Hansen and G.A. Spengler, (ed.). Lectins, Vol. III. Walter de Gruyter and Co., Berlin.

SUMMARY

The attachment to and infection of clover root hairs by Rhizobium trifolii are specific processes which are highly regulated (4). The regulation can be at the plant level, as exemplified by the inhibition of bacterial attachment and infection of clover root hairs when seedlings are grown with 15.0 mM KNO_3 (2). The culture age of R. trifolii also affects its ability to interact with trifoliin A, a white clover lectin (6). A third level of regulation involves interactions between clover and R. trifolii in the root environment, such as plant enzymes which degrade the capsular polysaccharide (CPS) of R. trifolii (5). This dissertation first examines properties of trifoliin A purified from clover seeds and seedling roots. Next, the effect of nitrate supply on the synthesis and distribution of trifoliin A was investigated. Lastly, the mechanism by which the culture age of R. trifolii 0403 affects the ability of trifoliin A to interact with the CPS was examined.

In Chapter 1, improved procedures for the purification of trifoliin A and measurement of its activity are described. The modified purification procedure requires half the time to isolate more than twice the amount of trifoliin A from seed meal than was obtained by the previous procedure (7). In addition, any quantity of clover seed can be processed, limited only by the size of the DEAE-Sephadex column. The ability to isolate larger quantities of lectin made it possible to characterize trifoliin A, most notably its amino acid and glycosyl composition, which required relatively large amounts of lectin (e.g., 1 mg protein for determination of the sugar components). Knowledge of the physical

nature of trifoliin A opens new doors for investigating the role this lectin plays in clover seeds and roots, how it is physically related to other legume lectins, and how one can proceed to further study trifoliin A itself.

The microtiter procedure developed for measuring R. trifolii lectin-binding activity has several advantages over the previously used tube agglutination method (7). Besides requiring less lectin and fewer cells, the progress of agglutination can be continually observed without disturbing the bacteria. This allows the maximum titer to be observed since the time for optimal agglutination varies depending on the bacterial culture, culture age, and any variations in the bacterial washing procedures. The endpoint titer is also easier to recognize with the microtiter plate method. The assay has been optimized by defining the optimal pH and cation requirements for bacterial agglutination.

The effect of nitrate supply on the synthsis of trifoliin A was investigated in Chapter 2. Several significant results were found. First, it was established that trifoliin A is synthesized in clover roots and that synthesis is not affected noticably by seedling growth with excess nitrate. However, the activity of the root exudate trifoliin A from seedlings grown with 15.0 mM nitrate was greatly reduced, as was the amount of trifoliin A on the root surface. These findings substantiated results from previous reports (2, 3). In addition, by combining in vivo incorporation of labeled amino acids to measure lectin synthesis with western blot analysis to determine total lectin, it was determined that the seed and root lectin appear to have very different functions. The seed lectin appeared to be degraded in the shoots of young seedlings, where little new synthesis of trifoliin A occured. This suggests that seed lectin acts as a storage protein providing the germinating seedling with amino acids during this heterotrophic stage of growth (1). The newly synthesized root lectin is excreted into the root environment rather than degraded during early seedling growth.

This, of course, is necessary if the lectin is to interact with R. trifolii during the early stages of the infection process. This finding, along with the result in Chapter 1 that root trifoliin A is much more active (16-fold) in binding to R. trifolii than the seed lectin, suggests that there are differences in the root and seed lectin in function and regulation. It is possible that the basis for these differences occurs at the genetic level, as has been shown for soybean lectin, in which the seed and root lectin are coded by different genes apparently under different regulation (8).

The capsular lectin receptor of R. trifolii 0403 was investigated in Chapter 3. It was established that the ability of R. trifolii to attach to clover root hairs is not merely due to the presence of a capsule, but to capsular trifoliin A receptors which change in distribution with culture age. The distribution, in turn, was found to affect the number of R. trifolii which attach to clover root hairs and the orientation of that attachment. Most importantly, these changes were correlated with chemical changes in the isolated CPS of R. trifolii 0403 at different culture ages. Although the sugar composition remained relatively constant, the degree of non-carbohydrate substitutions varied with culture age. A direct relationship was found between the acetate and pyruvate levels and the ability of the CPS to interact with trifoliin A.

The chapters in this dissertation describe some properties of trifoliin A and investigations of two mechanisms by which the interaction between R. trifolii and trifoliin A are regulated. While perhaps raising more questions than are answered, this work provides basic information into the functions of trifoliin A in clover, and the mechanisms by which the interactions between trifoliin A and R. trifolii are regulated.

In addition to the work presented in this dissertation, I have also been involved in several other projects. These included a study of plant enzymes in

the root exudate which degrade the capsular polysaccharide of R. trifolii (5). Another study demonstrated two phases of attachment of R. trifolii to clover root hairs, the first a loose, lectin-mediated attachment and the second an irreversible anchoring of the bacterium to the root hair by microfibrils (F.B. Dazzo, G.L. Truchet, J.E. Sherwood, E.M. Hrabak, M.Abe, and H.S. Pankratz, submitted for pulication). Trifoliin A has also been found in a particulate form in root exudate, which binds to the capsular polysaccharide but prevents the attachment of R. trifolii to the root hairs (G.L. Truchet, J.E. Sherwood, and F.B. Dazzo, manuscript in preparation). I was also involved in a project in which bacteriophage-induced polysaccharide lyases were used to depolymerize the capsular and extracellular polysaccharides of R. trifolii 0403 (R.L Hollingsworth, M. Abe, J.E. Sherwood, and F.B. Dazzo, submitted for publication). These oligosaccharides were demostrated to bind trifoliin A and stimulate the infection of clover root hairs by R. trifolii (M. Abe, J.E. Sherwood, R.L Hollingsworth, and F.B. Dazzo, submitted for publication).

List of references

1. Cooper, C.S. 1977. Growth of the legume seedling. Adv. Agron. 29:119-139.
2. Dazzo, F.B., and W.J. Brill. 1978. Regulation by fixed nitrogen of host-symbiont recognition in the Rhizobium-clover symbiosis. Plant Physiol. 62:18-21.
3. Dazzo, F.B., and E.M. Hrabak. 1981. Presence of trifoliin A, a Rhizobium-binding lectin, in clover root exudate. J. Supramolec. Struct. Cell. Biochem. 16:133-138.
4. Dazzo, F.B., and J.E. Sherwood. 1983. Trifoliin A: a Rhizobium-recognition lectin in white clover roots. pp. 209-223. In Chemical taxonomy, molecular biology, and function of plant lectins. L.J. Goldstein and M.E. Etzler, eds. A.R. Liss, Inc., New York.
5. Dazzo, F.B., G.L. Truchet, J.E. Sherwood, E.M. Hrabak, and A.E. Gardiol. 1982. Alteration of the trifoliin A-binding capsule of Rhizobium trifolii 0403 by enzymes released from clover roots. Appl. Environ. Microbiol. 44:478-490.
6. Dazzo. F.B., M.R. Urbano, and W.J. Brill. 1979. Transient appearance of lectin receptors on Rhizobium trifolii. Curr. Microbiol. 2:15-20.
7. Dazzo, F.B., W.E. Yanke, and W.J. Brill. 1978. Trifoliin: a Rhizobium recognition protein from white clover. Biochim. Biophys. Acta 539:276-286.
8. Goldberg, R.B., G. Hoschek, and L.O. Vodkin. 1983. An insertion sequence blocks the expression of a soybean lectin gene. Cell 33:465-475.