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Characterization of a Lectin from *Lactarius deterrimus*

Research on the Possible Involvement of the Fungal Lectin in Recognition between Mushroom and Spruce during the Early Stages of Mycorrhizae Formation

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A lectin (LDetL) was isolated from carpophores of the mushroom *Lactarius deterrimus*, a specific symbiont of the spruce, by a combination of affinity, hydroxylapatite, and gel-filtration chromatography. Its molecular mass, as determined by gel filtration, is about 37,000 D, and its structure is dimeric, with two identical subunits assembled by noncovalent bonds. It appeared homogeneous on high-performance liquid chromatography gel filtration, but isoelectric focusing revealed microheterogeneity, with a main band in the pH zone near 6.5. Amino acid analysis showed that LDetL contains a large proportion of glycine and especially methionine. Hapten inhibition assay indicated that LDetL is most specific for β-D-galactosyl(1→3)-D-*N*-acetyl galactosamine residues. The lectin was formed in the in vitro-cultivated mycelium, and anti-lectin antibodies revealed by indirect immunofluorescence the presence of lectin in the cell wall. Receptor sites for LDetL were found on the roots, especially on the root hairs, of axenically grown spruce seedlings. The lectin LDL previously isolated by us from the taxonomically related mushroom *Lactarius deliciosus*, a symbiont of the pine, does not bind to the spruce radicle. This suggests a role of the fungal lectin in recognition and specificity during the early stages of mycorrhizae formation.

Long thought to be restricted to certain groups, the symbiosis between green plants and other organisms such as bacteria, actinomycetes, and fungi has, in recent years, been shown to be extremely widespread. Of these mutually beneficial partnerships, the mycorrhiza, an association of a green plant with a fungus, is vital to the plant, which on its own cannot usually take up from the soil certain essential minerals, particularly phosphorus. Interest in these symbiotic associations and in the possibility of improving them to increase yields of crops and forest trees has prompted much work. Studies have revealed different types of mycorrhizae and shed light on the anatomy of these associations, their physiology, and their roles in water and mineral ion movement (Martin and Hilbert, 1991).

However, the recognition mechanisms between the partners have not been thoroughly investigated. Association does not occur by chance; both the plant and the fungus select one or more associates among the complex population of the rhizosphere, sometimes with a remarkably high degree of specificity. The problem of recognition extends well beyond the field of symbiosis; analogous if not identical mechanisms are doubtlessly involved in parasitic associations (Elad et al., 1983) and in the assembly of cells within a particular organism during embryogenesis (Levi and Teichberg, 1990).

Of the various explanations put forward to account for cell recognition and its specificity, the involvement of lectins seems highly probable. Lectins have been known for a century or so; they are synthesized by numerous plants and animals and play ill-defined but probably multiple roles in the physiology of the organisms that produce them. They can simply be reserve substances in some cases; in others they can regulate carbohydrate metabolism, act as a means of defense against parasites (Kojima and Uritani, 1974), or serve as intermediates in the action of hormones (Griffaut et al., 1990). A common feature is that they all bind more or less reversibly to glucoside residues. This ability, which calls to mind the specific interactions of the enzyme-substrate or antibody-antigen type, could account for affinity between host and symbiont, if one of them possesses a lectin on a membrane or wall and the other a complementary structure.

In the field of plant symbiosis, the role of lectins has been studied mostly in associations between higher plants and nitrogen-fixing microorganisms. After research (Hamblin and Kent, 1973) had shown that bean lectin could bind to a microsymbiont, the Rhizobium-Leguminosae symbiosis was thoroughly studied (Dazzo and Truchet, 1983; Kijne et al., 1986); in some legumes, for example, soybean (Halverson and Stacey, 1985), it seems that a lectin synthesized by the plant may play a role in recognition.

More recently, two other models have been investigated: the actinomycorrhiza and symbiosis involving cyanobacteria. An actinomycorrhiza is an association that occurs in ligneous plants whose root nodules are invaded by soil microorganisms of the actinomycetes. This symbiosis is important in the ecology of trees and shrubs that often grow as pioneers on nitrogen-deficient soil. Such trees and shrubs are of po-

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Abbreviations: FITC, fluorescein isothiocyanate; D-Galβ1→3D-GalNAc, β-D-galactosyl (1→3)-D-*N*-acetyl galactosamine; LDetL, *Lactarius deterrimus* lectin; LDL, *Lactarius deliciosus* lectin; pI, isoelectric point.

tential value for the reforestation of degraded land, either alone or in conjunction with other species that derive secondary benefit from the nitrogen fixation.

The actinomycetes species are all *Frankia*, but the corresponding symbiotic plants are distributed among several relatively primitive families of Dicotyledoneae, belonging to the orders Casuarinales (Callaham et al., 1978), Myricales, Fagales, Rhamnales, Coriariales, and Rosales (Dommergues et al., 1985). The sugars of the cell surfaces of the *Frankia* may contribute to specificity, because they differ according to whether the strains are isolated from *Alnus* or *Eleagnus* (Chaboud and Lalonde, 1983). Some cyanobacteria are involved in remarkable associations through their 2-fold ability to fix atmospheric nitrogen and carry out photosynthesis. These prokaryotes are hosted either by fungi in the lichen symbiosis or in vascular plants in associations of the *Anabaena-Azolla* (Peters et al., 1982) or *Nostoc-Gunnera* (Schaede, 1951) type. Studies on various lichens (Lockhart et al., 1978; Petit, 1978; Petit et al., 1983) led to the isolation of a lectin that apparently belonged to the fungal partner and that might be able to recognize structures on the cyanobacterium wall; the physiological relationship of the two partners may be modulated by variations in the sugars present on the wall of the phycobiont. Similarly, studies (Mellor et al., 1981; Ladha and Watanabe, 1984; McCowen et al., 1987) of the *Anabaena-Azolla* symbiosis have shown that a lectin belonging to the fern, and which differs according to the species, specifically recognizes the strain of cyanobacterium for which it adapted.

The occurrence of lectin activity in a large number of higher fungi (Coulet et al., 1970) and the existence of an often high degree of specificity in the associations of fungi with trees prompted us to determine whether the fungal lectins might be involved in the recognition of the symbionts. To this end, we chose as a model the milk cap mushrooms of the *Dapetes* group, all of which are associated with conifers, but which exhibit a remarkable specificity. Thus, the morphologically very similar *Lactarius deliciosus, Lactarius deterrimus,* and *Lactarius salmonicolor* are associated with the pine (*Pinus*), the spruce (*Picea*), and the fir (*Abies*), respectively.

We report here the results of a study of the involvement of the lectin in *L. deterrimus* in the recognition of the host spruce during the early stages of mycorrhizae formation. After isolating the lectin from the carpophores, we characterized its biochemical structure and specificity and compared it with the lectin previously isolated from *L. deliciosus*, the symbiont of the pine (Guillot et al., 1991). We also examined hyphae, particularly walls, for lectins, and the roots of young spruce seedlings for polysaccharide sites that might be recognizable by the fungal lectin.

MATERIALS AND METHODS

Fungal Material

Carpophores of *Lactarius deterrimus* were collected from beneath spruce trees in plantations in Chain of Puys near Clermont-Ferrand, France, and frozen at −18°C until use.

Isolation and Purification of Lectin

Pure samples of LDetL were obtained by three successive chromatographic separations. For preparation of crude extract, the carpophores were disrupted by the mean of an Ultraturax apparatus in 0.01 M phosphate buffer, pH 7.2 (PBS), at the ratio of 1:3 (w/v). The homogenate was kept at room temperature for 2 h and then filtered through a Tergal fabric (0.25 × 0.25 mm mesh). The extract was collected by centrifugation at 2000*g* for 10 min.

For affinity chromatography, group O human red blood cell stromas were incorporated into polyacrylamide gels according to a technique described previously (Bétail et al., 1975) and placed in a column (2.6 × 30 cm) maintained at 60°C. The column was loaded with 100 mL of the crude extract and then washed with PBS until the absorbance at 280 nm of the eluate was less than 0.01 (about 400 mL of PBS). The bound lectin was eluted with 200 mL of PBS buffer; readings and recordings were made with an LKB recorder. The eluates were collected in 10-mL fractions. To remove residual pigments and proteins contaminating the lectin thus obtained and to concentrate the eluate, it was further purified on hydroxylapatite. The lectin-containing fractions desorbed from the affinity column were dialyzed against phosphate buffer, pH 6.8 (1 mM sodium phosphate, 0.1 M NaCl), and then applied onto a column of hydroxylapatite (20-mL bed volume) equilibrated with the same buffer. The column was washed with 60 mL of the same buffer, and then the lectin was eluted with a linear gradient (100 mL) of phosphate buffer (1 mM to 0.5 M) in 0.1 M NaCl. Fractions of 5 mL were collected. As a control for the efficiency of the last step of purification, the lectin obtained by hydroxylapatite chromatography was subjected to HPLC gel filtration. It was dialyzed against 1 mM phosphate buffer, pH 6.8, and then applied at 25-μL fractions to a Waters Protein-Pak column at a flow rate of 0.5 mL min^{-1} at a pressure of 500 psi.

Hemagglutination

Hemagglutination assays were conducted by the method of Rosenfield and Haber (1965) with a Technicon autoanalyzer and a suspension of human red blood cells from a pool of at least 10 donors, at a concentration such that a transmission value of about 18% was obtained.

Inhibition of Hemagglutination by Sugars and Glycoproteins

For assaying the inhibition of hemagglutination, solutions of different sugars were placed in the sample rack of the Technicon autoanalyzer, and the agglutinating fractions were introduced continuously at a concentration that would cause agglutination of 50% of the O group cells. Each sugar was mixed with the agglutinin about 10 s before coming into contact with the red cell suspension. The concentration of sugar in each reaction medium was 50 and 33 mM after the addition of lectin and red blood cells, respectively. To elucidate the anomeric specificity and binding characteristics of the lectin, we tested various sugars related to *N*-acetyl-D-galactosamine. Because of their low solubility, they were dissolved in DMSO diluted 3-fold with PBS before use under the same conditions described above. The assay for inhibition of hemagglutination by glycoproteins was conducted in the same way as that for inhibition by sugars.

Characterization of LDetL

The mol wt of LDetL was determined by gel filtration on a Waters Protein-Pak 125 column (7.8 × 300 mm) equilibrated with 1 mM phosphate buffer and eluted with the same buffer at a flow rate of 0.5 mL min^{-1} at 20°C. The mol wt of the lectin was estimated by comparison of its availability coefficient (K_{AV}) with those of mol wt markers. SDS-PAGE was carried out in a 7.5% polyacrylamide gel that contained 0.1% SDS. The thin layer gels (250 × 11 × 2 mm) were polymerized in a glass polymerization chamber (LKB system). Electrophoresis was performed at 150 V for 5 h, and the proteins were stained overnight with Coomassie brilliant blue R-250. IEF was performed on a 1% agarose gel containing 12% sorbitol and 2% ampholites selected to establish a pH gradient from 3 to 10. The layer gels were prepared in a glass chamber (11.5 × 11.5 × 0.04 cm). Protein concentration was estimated according to Bradford (1976) with BSA as a standard. The phenol-sulfuric acid method (Dubois et al., 1986) was used to examine the possible presence of a carbohydrate moiety on the lectin. The amino acids resulting from acid hydrolysis of the protein were converted into their phenylthiocarbamylate derivatives in the presence of phenylisothiocyanate and triethylamine, which ensures an alkaline pH. The derivatives prepared in this way were then separated on a C_{18} grafted silica column by reverse-phase HPLC and detected and assayed by their absorption at 254 nm according to a technique reported previously (Guillot et al., 1991).

In Vitro Culture of the Mycelium of *L. deterrimus* and Extraction of the Lectin from the Mycelium

Fragments (5 mm wide) were obtained aseptically from young carpophores. After five successive washings for 15 min each in 70% ethanol, they were rinsed for 15 min five times with sterile distilled water and then placed in a bottle containing 10 mL of Pachlewski medium (Pachlewski and Pachlewska, 1974) for preculturing. The mycelium that grew in about 1 week of culture under constant shaking at 20°C in the dark was subcultured in 400 mL of the same medium. The culture conditions were as described above. The mycelium was harvested after 4 to 6 weeks and washed several times in PBS buffer to remove culture medium. The lectin was extracted by grinding in a mortar after freezing and then subjected to the first stage of the purification, chromatography on erythrocyte stromas.

To check for parietal localization of lectin, we applied an enzyme treatment to part of the mycelium obtained by culture, using a technique advocated by Ishikawa et al. (1983). Samples of mycelium (0.5 g) were defatted with an equal amount of a mixture of chloroform (2 volumes) and methanol (1 volume) and then incubated with the different enzymes in a 66-mM phosphate buffer. The enzymes were cellulase from *Trichoderma viride*, chitinase from *Streptomyces griseus*, and lyticase (or zymolase), an endo-β-1,3-glucanase from *Arthrobacter luteus*. Incubation conditions were as follows: 15 h at 37°C at pH 5 for the cellulase (100 units), 48 h at 25°C at pH 6 for the chitinase (136 units), and 15 h at 25°C at pH 7.5 for the lyticase (2.15 units). Lectin activity was evaluated in the culture medium by hemagglutination using serial dilutions after concentration in a dialysis tube over PEG 35,000.

Anti-Lectin Polyclonal Antibodies

Anti-lectin polyclonal antibodies were obtained by immunization of female white hybrid rabbits. Blood taken on heparin from the auricular artery was washed three times in sterile phosphate buffer and centrifuged at 150*g* for 10 min. The pellet was resuspended at a concentration of 4% (v/v) in the same buffer. Two drops of this cell suspension were added at 0.5-mL solutions obtained by successive 2-fold dilutions of lectin in buffer. The agglutinates in the four highest dilutions were pooled, washed three times in sterile buffer with centrifugation each time, and resuspended in 1 mL of buffer. The suspension of sensitized cells was then reinjected into the marginal vein of the ear of the same rabbit. This operation was repeated weekly for 20 weeks. Concomitantly, about 10 mL of blood was sampled to recover the immune serum; this was divided into 1-mL fractions and stored at −20°C.

An immune serum containing antibodies against LDL was obtained in the same way. The method of Ouchterlony (1953) was used for the observation of the rise in antibody level and for the immunological comparison of the lectins derived from the carpophore and from the mycelium obtained by culture.

Tests for Parietal Lectin Expressed on Mycelial Hyphae

Both fresh material and sections of deep-frozen fixed mycelium were examined. In the first case, the mycelium was first washed three times in PBS buffer to remove all traces of medium and then twice in PBS buffer containing 2% BSA (PBS-BSA). It was then placed in contact with the rabbit immune serum in a range of dilutions in PBS-BSA. Identical control series of dilutions were prepared with nonimmune rabbit serum and immune serum containing antibodies against LDL. After 1 h of incubation, the mycelium was washed three times in PBS-BSA and treated for 1 h in the dark with a 100-fold diluted solution of goat anti-rabbit immunoglobulin antibodies that had been labeled with FITC. The mycelium was then washed twice with PBS-BSA and once with PBS before it was examined by fluorescence microscopy.

For examination of fixed sections, fragments of mycelium of about 1 mm^3 were rinsed with phosphate buffer, fixed with paraformaldehyde by incubation at 4°C for 2 h, thoroughly washed to remove all fixer, and dehydrated by three successive 15-min immersions in 50% ethanol and then one immersion in 70% ethanol. The sections obtained after deep-freezing the samples were mounted on slides and rinsed three times with PBS, twice with PBS containing 50 mM NH_4Cl, and twice with PBS containing 2% BSA. Dilutions of rabbit immune serum were made in PBS and placed in contact with the preparations for 1 h under high humidity at room temperature. The sections were rinsed three times in PBS-BSA and then treated for 1 h in the dark under high humidity at room temperature, with a solution of goat anti-rabbit antibodies labeled with FITC diluted 100-fold in PBS-BSA.

Analysis of Spruce Seedlings for Lectin Receptors

Seeds of *Picea abies* were left in distilled water overnight and then in 30% hydrogen peroxide solution for 6 h. They

were then transferred under sterile conditions to Petri dishes containing Sabouraud agar medium and left in the dark at room temperature until rootlets appeared, i.e. about 8 d. At this initial germination stage, the seeds were placed under aseptic conditions in 2-L jars one-third filled with vermiculite impregnated with 400 mL of Shemakhanova medium (Shemakhanova, 1967) per L of vermiculite. The cultures were left to grow under the following conditions: temperature 22°C; illumination 18 W/m^2 provided by Mazda LDL TF 40-W tubes; humidity 60%; light-dark cycle with 16 h light and 8 h dark. The seedlings were harvested between 1 and 6 weeks later.

Two methods were used to examine spruce roots for lectin receptors: (a) a direct method in which fluorescein-labeled LDetL was placed in contact with the roots, and (b) an indirect method involving detection by immunofluorescence.

Direct Method

The lectin was coupled with FITC by the technique of Goldman (1968) and isolated by gel filtration on Sephadex G-25. Because the fluorochrome coupling made colorimetric and photometric assay impossible, the concentration of the labeled lectin was deduced from its hemagglutinating titer compared with that of the unlabeled lectin; unlabeled lectin concentration was estimated by the method of Bradford (1976). After sampling, the roots were washed in Shemakhanova medium and immersed for 1 h at 4°C in the dark in the lectin solution, the titer of which was 1/512 exprimed in hemagglutinating activity as measured by the method of successive 2-fold dilutions. They were then rinsed with the medium and examined by fluorescence microscopy.

Indirect Method

The roots were washed in Shemakhanova medium and placed in a solution containing 2 μg of lectin mL^{-1} for 1 h at room temperature in the dark and then washed three times for 5 min each with Shemakhanova medium supplemented with 2% BSA. They were then placed in contact with successive dilutions of the primary antibody (rabbit immune serum) for 1 h and then, after washing three times for 5 min, placed in contact with the secondary antibody (goat anti-rabbit antibody labeled with FITC) diluted 100-fold. After rinsing twice with Shemakhanova medium supplemented with BSA, then Shemakhanova medium alone, samples were prepared for examination in equal volumes of glycerin and Shemakhanova medium. Three control series (without lectin, without primary antibody, and without secondary antibody) were run concomitantly. The same procedure was applied to the LDL and the corresponding immune serum.

RESULTS

Isolation and Purification of LDetL

From 100 mL of crude extract, affinity chromatography on erythrocytal stromas yielded about 150 mL of solution containing a hemagglutinating activity (Fig. 1). The lectin could be completely eluted by a 0.1-M solution of *N*-acetyl-D-galactosamine; however, we chose to elute by raising the temperature. Preliminary experiments have shown that this sugar has the disadvantage of partially and irreversibly binding to the lectin and that the dialysis required to remove the sugar causes some loss of lectin activity.

Filtration on hydroxylapatite proved efficient for removing contaminants, particularly pigments, and enabled the lectin to be concentrated about 2-fold. HPLC gel filtration eliminated only insignificant traces of impurities of low mol wt and gave a sharp symmetrical peak (Fig. 2), showing LDetL to be homogeneous. HPLC gel filtration was used to obtain a pure protein for the assay of subunits and pI. This technique was also used for the calculation of the mol wt. A summary of a typical purification of LDetL is shown in Table I. About 0.16 mg of the purified lectin was obtained from 25 g of mushrooms, with a yield of 99% in hemagglutinating activity.

Molecular Structure

Because of nonspecific binding between native lectin and polyacrylamide gel, the mol wt of LDetL was determined by gel filtration. This method gave a molecular mass of about 37,000 D (Fig. 2). Analysis of LDetL by SDS-PAGE in the presence and absence of mercaptoethanol showed a single band with a molecular mass of about 18,000 D. These results show the lectin to be a protein dimer composed of two identical subunits assembled by noncovalent bonds (Fig. 3). Electrofocusing revealed a microheterogeneity, with a main band in a pH zone near 6.5 (Fig. 4). The amino acid composition of LDetL is shown in Table II. Gly and especially Met are present in large amounts. No carbohydrate was detected in the molecule by the method of Dubois et al. (1986).

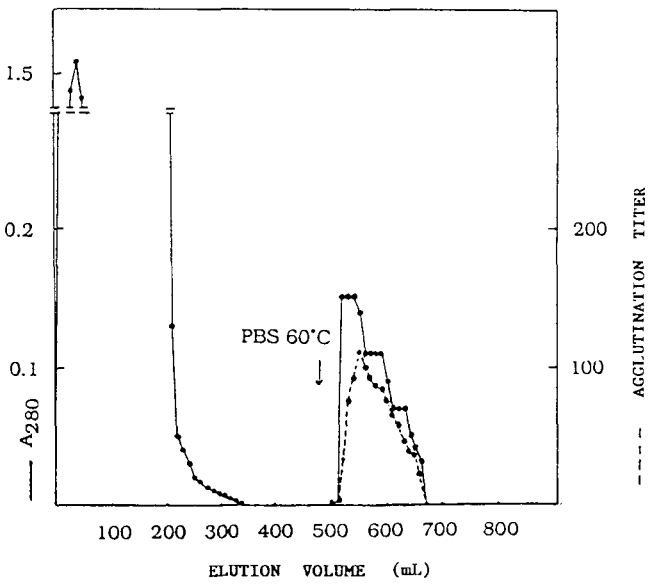

Figure 1. Affinity chromatography on group O human red blood cell stromas. The column was loaded with 100 mL of the crude extract and washed with 400 mL of PBS. The fixed lectin was eluted with 200 mL of PBS. The column was enclosed in a temperature-controlled jacket to maintain a constant temperature of 60°C throughout the operation. The eluates were collected in 10-mL fractions.

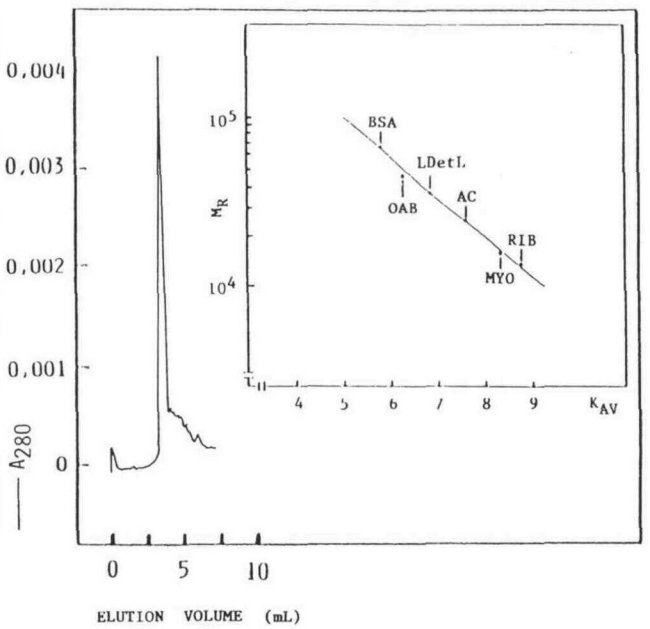

Figure 2. HPLC gel filtration chromatography of the hydroxylapatite-purified lectin on a column of Protein-Pak SW 125. The inset shows the determination of M_r by gel filtration of LDetL on the same HPLC column. The mol wt markers were ribonuclease A (RIB, 13,700); myoglobin (MYO, 17,000); carbonic anhydrase (AC, 29,000); ovalbumin (OAB, 43,000); and BSA (67,000).

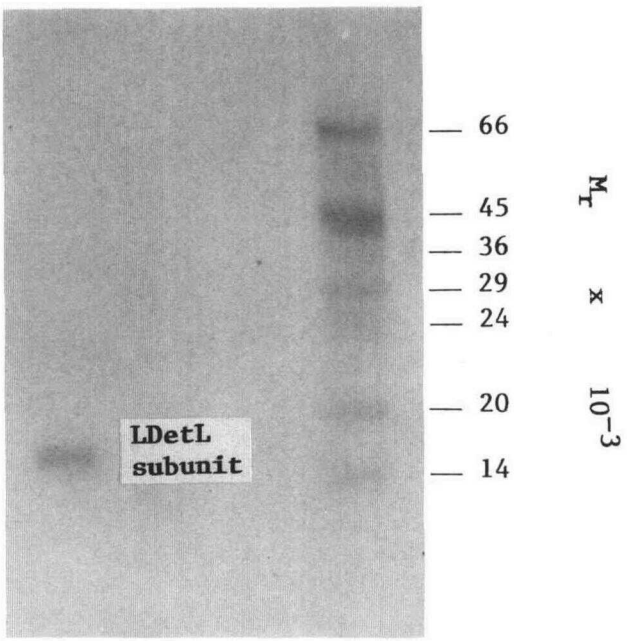

Figure 3. SDS-PAGE of LDetL. The mol wt markers were α-lactalbumin (14,200); soybean trypsin inhibitor (20,100); trypsinogen (24,000); carbonic anhydrase (29,000); glyceraldehyde-3-phosphate dehydrogenase (36,000 [subunit]); ovalbumin (45,000); and BSA (66,000). With or without mercaptoethanol, LDetL behaved as a single band at about 18,000 D.

Biological Activity

The lectin is not specific with regard to any particular erythrocyte phenotype in the ABO system; red cells of all phenotypes were agglutinated to comparable extents. Only *N*-acetyl-D-galactosamine and related sugars and glycoproteins, particularly the disaccharide β-D-Gal(1→3)D-GalNAc, inhibited the lectin (Table III). The hemagglutinating activity of LDetL was not inhibited by any of the following sugars chosen from the four groups of Mäkelä (1957): D-arabinose, L-fucose, and D-ribose (group I); L-arabinose, D-galactose, D-galactosamine, α-lactose, melibiose, stachyose, raffinose, and *N*-acetyllactosamine (group II); D-cellobiose, D-fructose, D-glucose, D-glucosamine, *N*-acetyl-D-glucosamine, D-lyxose, maltose, D-mannose, sucrose, trehalose, and D-xylose (group III); L-glucose, L-rhamnose, L-sorbose, and L-xylose (group IV).

Glycoprotein concentrations (in μg mL^{-1}) that inhibited agglutination by 50% were 0.1 (asialo-bovine submaxillary mucin); 0.9 (bovine submaxillary mucin); 26 (asialofetuin); 200 (fetuin); 400 (α_1-acid glycoprotein); 7000 (human transferrin).

Evidence for and Localization of Lectin from Mycelium Cultivated in Vitro

Four hundred milliliters of culture medium, concentrated by dialysis against PEG followed by PBS to obtain 1 mL of

Table I. *Purification of LDetL from 25 g of mushrooms (L. deterrimus)*

Hemagglutination assays were conducted by a method using an autoanalyzer apparatus with a suspension of human O group red blood cells. One unit of hemagglutinating activity is defined as the amount of lectin that is able to eliminate 0.1 g of hemoglobin in the experimental conditions.

Fraction	Total Protein	Total Hemagglutinating Activity	Specific Hemagglutinating Activity	Protein Recovery	Hemagglutinating Activity Recovery
	mg	*units*	*units mg*$^{-1}$	%	%
Crude extract with PBS	42	1050	25	100	100
Affinity chromatography	1.6	1040	650	3.8	99
Hydroxylapatite chromatography	0.16	1035	6468	0.38	98.5

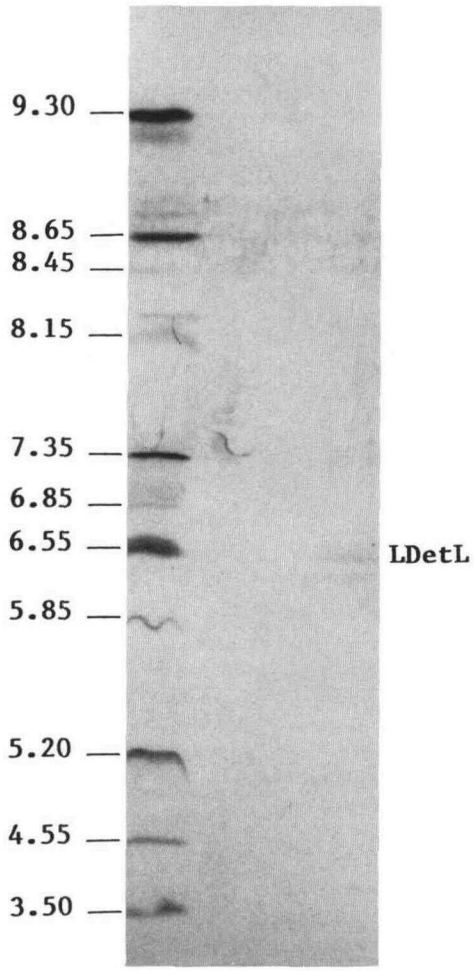

Figure 4. IEF in a 1% agarose gel with a pH gradient of 3 to 10. The standard proteins for pI determination were amyloglucosidase (3.50); soybean trypsin inhibitor (4.55); β-lactoglobulin A (5.20); bovine carbonic anhydrase B (5.85); human carbonic anhydrase B (6.55); horse myoglobin (7.35); lentil lectin I (8.15); lentil lectin II (8.45); lentil lectin III (8.65); and trypsinogen (9.30). Electrofocusing revealed microheterogeneity, with a main band in a pH zone near 6.5.

solution, contained a lectin that agglutinated O-group red cells with a titer of 1/16, as measured by the method of successive 2-fold dilutions. Grinding 1 g of frozen mycelium yielded 0.2 mL of a lectin with a titer of 1/4. Treatment of the mycelium with chitinase and cellulase had no effect, but the action of lyticase caused the release of a fairly large amount of lectin; 1 mL of solution of titer 1/250 was obtained from 1 g of mycelium.

Comparison of the mycelial lectin with that isolated from the carpophore by the method of double diffusion in a gel in the presence of anti-carpophore lectin antibody shows them to be immunologically identical. The double-labeling method applied to fresh mycelium or mycelium sections revealed a marked fluorescence on the walls of the mycelial hyphae by light microscopy (Fig. 5).

Fixation of LDetL on Spruce Rootlets

A kinetic study of the evolution of the radicles of spruce seedlings showed that the root hairs appear after 1 week and the secondary roots 2 weeks after potting.

Fixation of FITC-labeled lectin was mostly restricted to the root hairs and the tips of the young secondary rootlets (Fig. 5). The root surface proper and the tissues undergoing suberization seemed to possess markedly fewer lectin receptor sites. This observation was confirmed indirectly; fluorescence was observed up to an immune serum dilution of 1/1000 for the root hairs and secondary rootlets, but less strongly for the pileorhiza (Fig. 5). Both methods showed that placing the lectin in contact with D-GalNAc solution inhibited its subsequent fixation. Also, control runs with preimmune serum and with LDL were negative.

DISCUSSION

Because of its specificity, we chose the *L. deterrimus–P. abies* association to establish that one of the first steps of the mycorrhiza symbiosis is a mutual molecular recognition. The molecules implicated in the recognition are: (a) a lectin produced by the mushroom and expressed at the surface of the hyphae, and (b) polysaccharide moieties on the cell walls of the radicle.

First, we obtained evidence for the existence of a lectin produced by the mycelium of the mushroom grown in vitro. Because mycorrhizal synthesis in vitro had already shown

Table II. *Amino acid composition of LDetL*

The amino acids resulting from acid hydrolysis of the protein (100 μL of solution containing 15 μg of lectin) were converted into their phenylthiocarbamyl derivatives in the presence of phenylisothiocyanate and triethylamine. The derivatives then were separated on a C_{18} grafted silica column by reverse-phase HPLC and assayed by their absorption at 254 nm. Values are the means of those obtained with two different hydrolysates. The mol wt of LDetL was assumed to be 37,000. Tryptophane was not determined.

	Amino Acid Residues	
	g 100 g⁻¹ protein	*residues molecule⁻¹*
Lysine	4.73	13.6
Histidine	1.95	5.5
Arginine	5.13	12.2
Aspartic acid	5.55	17.8
Threonine	6.82	18.5
Serine	5.88	24.9
Glutamic acid	8.85	25.3
Proline	2.20	8.4
Glycine	6.17	40.0
Alanine	2.50	13.0
Half-cystine	Traces	Traces
Valine	4.48	16.7
Methionine	26.49	74.7
Isoleucine	3.44	11.2
Leucine	5.90	19.3
Tyrosine	5.37	12.2
Phenylalanine	4.54	11.4

Table III. *Inhibition of agglutination of group O human red cells by LDetL*

The inhibition of hemagglutination by sugars was monitored by autoanalyzer with a suspension of group O human blood cells. Lectin was introduced continuously at a concentration that would cause agglutination of 50% of cells present. Each sugar was mixed with the lectin 10 s before coming into contact with red blood cells. The concentration of sugar was 50 and 33 mM after the addition of lectin and cells, respectively. Inhibition activity is expressed in percent.

Sugar	Sugar Concentration (mM)								
	100	25	12.5	6.5	1	0.5	0.25	0.125	0.0625
D-GalNAc	16.4	0							
D-GalNAc 1P		11.3	0						
o-Nitrophenyl α-D-GalNAc		0							
p-Nitrophenyl α-D-GalNAc		0							
p-Nitrophenyl β-D-GalNAc		—[a]	0						
D-Galβ1→3D-GalNAc		100	100	100	100	39	16	4	0
D-Galβ1→3D-GlcNAc		16	0						
D-Galβ1→4D-GlcNAc		0							
D-Galβ1→6D-GlcNAc		0							
Pneumococcus type XIV polysaccharide		0							

[a] Insoluble at this concentration.

that the mycelial elements grown in an artificial medium have the same infective abilities as those in the soil, it could be assumed that the molecules involved in the early stages of mycorrhizae formation were present in both. A lectin activity was detected in the culture medium, and a lectin was released by enzyme action from the wall of the mycelial hyphae. The localization of a lectin on the mycelium surface has been reported for other species of fungus; this superficial location enables it to play a role in recognition processes. A lectin situated on the hypha wall was reported by Ishikawa et al. (1983) in *Conidiobolus lamproges*. In this species, the lectin seems to be involved in the early adhesion of the fungus to the cuticle of various insects. Similarly, *Rhizoctonia solani* has on the surface of its wall a lectin that is involved in a mycoparasitic relationship with *Trichoderma harzianum* (Elad et al., 1983).

Because the amount of lectin released from the mycelium was too small for its biochemical characteristics and specificity to be determined, we isolated the soluble lectin that was present in appreciable amounts in the carpophore. It was shown to be a protein of 37 kD, composed of two identical subunits linked by noncovalent bonds. Comparison of LDetL with LDL showed similar features but also significant differences. Both lectins have mol wts near 38,000, a dimer-type structure with subunits held together by noncovalent bonds, and a pI close to 6.5. However, unlike LDetL, LDL has nonidentical subunits (type A_1B_1) and a particularly high Gly content (Guillot et al., 1991). LDetL can bind to residues of the type β-D-Gal(1→3)D-GalNAc and to a lesser degree to D-GalNAc alone; it is also inhibited, although less so, in hemagglutination reactions by the disaccharide β-D-Gal(1→3)D-GlcNAc, although the component monomers are not active. LDetL is similar in specificity to the anti-T LDLs (Guillot et al., 1991) and various Leguminosae including *Arachis hypogaea, Maclura pomifera, Bauhinia purpurea,* and *Sophora japonica*. LDetL, which is inhibited by D-GalNAc but not by *N*-acetyllactosamine, has binding characteristics similar to those of lectin of *Maclura* (Wu, 1984). This specificity is confirmed by experiments with glycoproteins, desialylated fetuin being the most active.

Polyclonal antibodies prepared from carefully purified carpophore lectin show that this lectin and that isolated from mycelium have the same immunological properties in a double immunodiffusion test and are thus doubtlessly very similar if not identical. In addition, these antibodies reveal, by indirect immunofluorescence, the presence of lectin in the wall of mycelium grown in vitro. The presence of surface lectin suggests that it may be involved in recognition of glycoconjugates.

The radicle systems of axenically grown spruce seedlings aged 1 to 6 weeks were exposed to LDetL and identified by a direct technique with FITC-labeled lectin or an indirect technique in which the fixed lectin is detected by anti-lectin antibodies. Both methods revealed fixation sites, the specificity of which was verified by inhibition with D-GalNAc. The lectin is fixed preferentially on young tissue: root hairs, pileorhiza and the area immediately above, and at the tips of secondary rootlets; its presence on the other surface cells is much sparser. In addition, LDL, which we isolated from *L. deliciosus*, is specific to the genus *Pinus*, although it has the same overall specificity as LDetL, is not fixed on the spruce radicle. It may be concluded that LDetL behaves as an exolectin (Gallagher, 1984), recognizing a complex glycoside sequence at the surface of plant tissues that is different from that present on pine roots.

Wu (1984) showed that lectins with overall β-D-Gal(1→3)D-GalNAc specificity had no common pattern affinity for other disaccharides and so differed in this respect. Similarly, Rougé et al. (1978), studying interactions between various lectins from Leguminosae and normal and pathological human serum glycoproteins, found differences in their accurate specificities. *Canavalia ensiformis* lectin and the lectins isolated

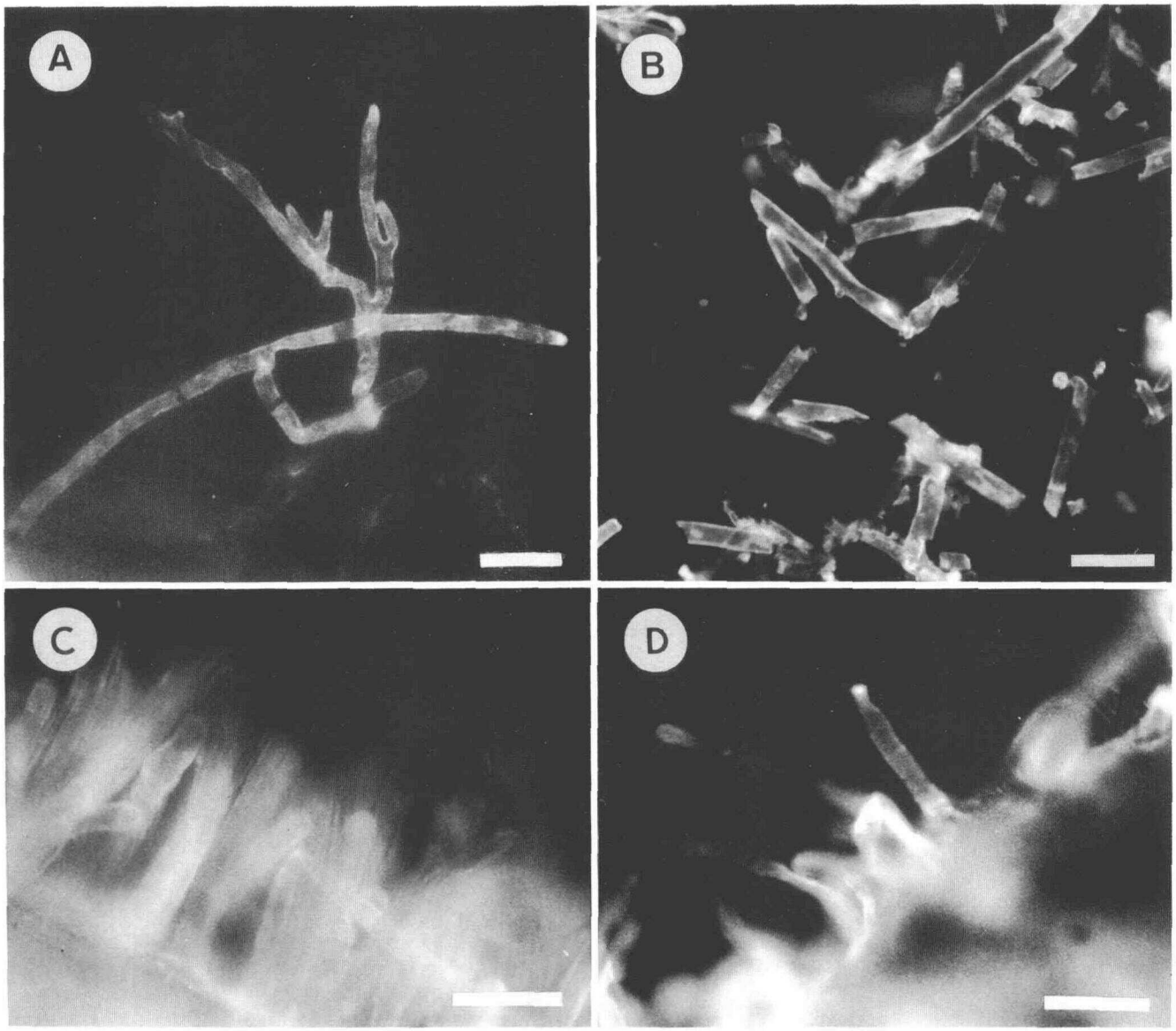

Figure 5. A and B, Labeling of mycelium of *L. deterrimus* cultured in vitro on Pachlewski medium. A, Fresh mycelium was placed in contact with an anti-LDetL rabbit immune serum dilution and then treated with a goat anti-rabbit immunoglobulin antibody labeled with FITC. B, Paraformaldehyde-fixed sections of deep-frozen mycelium were treated in the same way as above. In both cases, the double-labeling method revealed a marked fluorescence on the walls of the hyphae. Bar, 10 μm. C and D, Fluorescence photograph of spruce root with root hairs. C, After contact with the FITC-labeled LDetL. D, After contact, first with an anti-LDetL rabbit immune serum, then a goat anti-rabbit antibody labeled with FITC. Direct and indirect methods shows the presence of specific sites for the lectin on the roots hairs. Bar, 50 μm.

from the lentil, pea, and seeds of *Lathyrus tingitanus* are apparently identical in terms of overall specificity (all four are inhibited by Glc and Man and their derivatives); however, they show differences in reactivity, even between isolectins (haptoglobin reacts with only one of the two isolectins from pea seeds). These differences may be explained by slight variations in the conformation of carbohydrate fixation sites, doubtlessly originating in the mutations that have occurred in their primary sequences. The involvement of lectins of Leguminosae in the early stages of recognition between these plants and *Rhizobium* seemed doubtful at first because all the lectins had the same specificity within certain tribes. In fact, these specificities toward sugars are not identical; they require more accurate evaluation using glycoproteins or complex carbohydrates. Still, the carbohydrates present on the various species of *Rhizobium* must be different. Recent studies (Lerouge et al., 1990) have shown that nodulation factors secreted by *Rhizobium* after activation of *nod*

genes by flavonoids released by Leguminosae are lipooligosaccharides. All the species of *Rhizobium* produce the same basic structure: a chain of four or five *N*-acetylglucosamines linked β 1→4, with a fatty acid on the nonreducing end of glucosamine. However, each different species of *Rhizobium* is characterized by numerous variations on this common structure: the two terminal glucosamines bear specific groups, and the structure of the fatty acid differs between species. First, the *nod* genes common to all the *Rhizobium* species, the *nod* ABC genes, encode the enzymes that synthesize the basic structure of the nodulation factors; then the *nod* genes specific to individual *Rhizobium* species "decorate" this basic structure in a particular way. The lipooligosaccharide then binds with a receptor located on the root surface and activates early nodulin genes in the plant. This receptor is probably a lectin. In the same way, the lectins of *Lactarius* have related overall specificities, reflecting a common genetic origin; subsequent specific variations have led to an adjustment of the recognition sites to particular saccharide patterns.

The reactivities of LDetL and LDL toward GalNAc and β-D-Gal(1→3)D-GalNAc show marked differences. These sugars are much more active in hemagglutination inhibition reactions with LDL. Thus, the disaccharide β-D-Gal(1→3)D-GalNAc may have a structure that is closer to that of the LDL-complementary sugar than to that of the LDetL-complementary one.

Evidence for mechanisms of the lectin type has often been found in the related field of parasite-host associations, involving microsymbionts, bacteria or fungi, and both animals and higher plants (Hohl and Balsiger, 1986). Although lectin involvement in symbiosis has been extensively investigated in *Rhizobium*-Leguminosae associations, little work has been done on the molecular mechanisms responsible for recognition and specificity during mycorrhizae formation. In work on the ericoidal endomycorrhiza of *Pezizella ericae*, Gianinazzi-Pearson et al. (1986) discovered that the fungus secreted exocellular fibrils when grown axenically in the presence of a host plant, on first coming into contact with it. Although the authors suggest that this may be the first event in the cell recognition, it may be that the fibrils are only a secondary effect, as in the attachment of *Agrobacterium tumefaciens* (Matthysse, 1986). In the *L. deterrimus*–spruce model, the facts that there is a lectin at the surface of the mycelial hyphae and that this lectin corresponds to receptors on the cell walls of the host radicle are evidence for a highly specific lectin system resulting from the coevolution of the two associated species.

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