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Cephalodia of the Lichen *Peltigera aphthosa* (L.) Willd. Specific Recognition of the Compatible Photobiont

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Abstract

A specific lectin was isolated from cephalodiate thallus lobes of the lichen *Peltigera aphthosa* and purified to homogeneity by two steps: Chromatography on carboxymethyl-cellulose (CMC); and affinity chromatography on a lactose agarose column. TEM immunogold labeling using antibodies raised against the lectin, revealed a clear gold labeling of fungal cell walls adjacent to the cephalodial *Nostoc* cells. We suggest that the lectin located on the fungal cell wall recognizes the compatible *Nostoc* at the initiation of cephalodia formation.

Keywords: Lichens, *Peltigera aphthosa*, cephalodia, *Nostoc*, fungal hyphae, immunolabeling

1. Introduction

Peltigera aphthosa (L.) Willd. is a tripartite lichen containing one mycobiont and two photobionts. The green alga *Coccomyxa* forms the algal layer of the thallus lobes and the cyanobiont *Nostoc* is restricted to delimited areas on the thallus – the cephalodia.

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According to microscopic observations, even when various cyanobacteria or green algae are growing epiphytically on the thallus only the preferred cyanobiont is incorporated and the cephalodium formed (Jahns, 1988).

The surface of *P. aphthosa* is covered with fine-delicate fungal outgrowths, which presumably trap the cephalodial cyanobiont. The cortex and the algal layer beneath the cephalodia dissociate and a direct contact between the cephalodia and the internal part of the thallus is established (Jahns, 1988).

Paulsrud and Lindblad (1998) and Paulsrud et al. (1998) confirmed that only one cyanobiont was found in cephalodia on different parts of one thallus and on thalli from different localities by using the $tRNA^{Leu}$ (UAA) intron as a genetic marker. This implies that the acquisition and incorporation of the cephalodial *Nostoc* is a highly specific process and requires a very sensitive recognition mechanism.

The main object of this study is to test the mechanism of discrimination between the compatible and the incompatible cephalodial photobiont of *P. aphthosa*. For this purpose and in view of previous findings (Lockhart et al., 1978; Petit, 1982; Bubrick, 1984; Kardish et al., 1991) we tested the possible role of a lectin extracted from cephalodiate thallus lobes in the discrimination process.

2. Materials and Methods

Peltigera aphthosa (L.) Willd. was collected from Rondane, Norway (Fig. 1).

Extraction and purification of the lectin from P. aphthosa (designated PAA = Peltigera aphthosa agglutinin)

Five g (d.w.) of thallus lobes with cephalodia were homogenized with glass beads in a mortar with pestle with 50 ml phosphate buffered saline (PBS) (100 mM sodium phosphate buffer, pH 7.2) and 150 mM NaCl, containing 3 mM dithiothreitol (DTT), 1 µg/ml phenylmethylsulfonyl fluoride (PMSF), 15 mM sodium azide, 0.01% (v/v) Tween 80 and 3% (w/v) polyvinilpolypyrrolidone (PVPP), pretreated with 10% HCl to remove Cl^- (Loomis, 1974). The homogenate was stirred for 24 hr at 4°C and centrifuged at 20,000 g for 20 min. The supernatant was collected and stored at 4°C, until used.

The crude extract was dialysed for 16 hr at 4°C against 10 mM PB (sodium phosphate buffer, pH 7.2) and then purified by two chromatographic steps:

Ten ml of the dialysate were applied to a CMC (carboxymethyl-cellulose, Whatman) column (1.9×26 cm). The column was equilibrated with the same buffer as above and developed with a linear gradient of 0–2 M NaCl. A fraction

Figure 1. *Peltigera aphthosa* (L.)Willd.

possessing hemagglutinating activity was eluted from the column. This fraction was further purified by affinity chromatography on a lactose agarose column. Lactose agarose in suspension was equilibrated with PB containing 1 M NaCl. Four ml of the fraction obtained from the CMC column, to which NaCl was added to a final concentration of 1 M, were mixed with 5 ml of a lactose-agarose suspension and rotated for 16 hr at 4°C. The suspension was packed into a column (1×10 cm), washed with 25 ml buffer (as above) and eluted with 2 M urea. The fractions were dialysed against ddH_2O for 16 hr at 4°C, concentrated 5-fold in a Speed-Vac apparatus (Savant Instruments, Farmingdale, NY) and tested for hemagglutination.

Agglutination assays and sugar specificity assays were carried out as described by Lehr et al. (1995). Protein concentrations were measured according to Marder et al. (1986).

PAGE-SDS and native electrophoresis and immunoblotting

SDS-Page electrophoresis was performed as in Kardish et al. (1991) using 12.5% polyacrylamide gels. The gels were either stained with Coomassie Brilliant Blue (R250) or with silver or electrotransferred to nitrocellulose membranes. Electrophoresis on native gel-acrylamide was performed according to Laemmli (1970) except that a lower buffer of 63 mM Tris, 50 mM HCl, pH 7.74 and an upper buffer of 37.4 mM Tris, 40 mM glycine, pH 8.89 were used. The samples were prepared with 0.1% bromophenol and 50% sucrose. At the beginning the gel was run at 50 mV and then the voltage was lowered to 25 mV for 6 hr at 4°C.

Immunostaining

The transfer was for 1 hr and 15 min at 170 V in 12.5 mM ethanol/glycine buffer, pH 9.5, containing 20% methanol. The blot was stained with 0.1% Ponceau Red in 0.7% TCA. The lane containing the standards was cut out and the remaining blot washed with Tris-buffered saline (TBS) (200 mM NaCl, 50 mM Tris-HCl, pH 7.4) to remove the stain.

After eletrotransfere, the blots were blocked for 1 hr with 1% milk containing 0.02% sodium azide and incubated overnight at room temperature with the anti-lectin (see below) diluted 1: 20,000 or 1: 10,000 in the blocking solution while shaking in a shaker bath at 100 strokes/min followed by the procedure as in Lehr et al. (1995).

Preparation of antiserum

Antisera to the two closely migrating bands, (PAA1) and (PAA2) that were excised from the nitrocellulose membrane, were prepared. Approximately 75 µg of the protein were dissolved in 400 µl DMSO (dimethylsulfoxide) to which 400 µl of Complete Freund's Adjuvant (Difco Laboratories, Detroit, MI) was added for the first injection. Then approx. 75 µg of protein in Incomplete Freund's Adjuvant were used for 3 subsequent booster injections at 3 week intervals. The rabbits were bled from the ear 3 weeks after each booster injection. After 3 hr at room temperature and overnight at 4°C the blood was centrifuged (20,000 g for 30 min) and the supernatant stored at –20°C until used.

The antibodies raised against the more pronounced band (Fig. 2), which showed hemagglutinating activity were used for TEM immunolabeling.

PAA localization by transmission electron microscopy (TEM)

Cephalodiate thallus lobes prepared for thin section TEM observations were

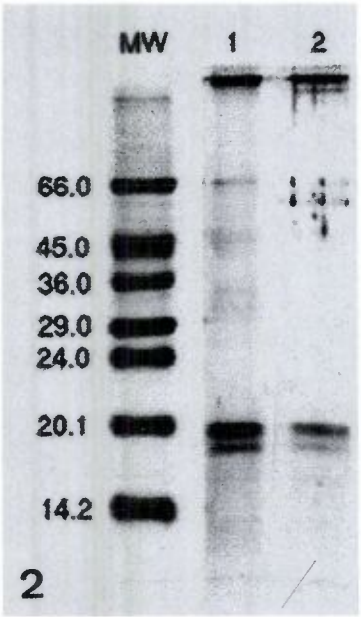

Fig. 2.

Fig. 3.

Figure 2. SDS-PAGE of the two-step purified extract of *Peltigera aphthosa* that revealed two closely migrating bands of approx. 20 kDa PAA1 and PAA2. Positions of molecular mass markers are indicated at the left.

Figure 3. PAA1 and PAA2 on a silver-stained native gel.

fixed in 0.5% glutaraldehyde, 2% paraformaldehyde and 0.5% DMSO in 0.05 M PB (pH 7.2), for 2 hr in vacuum and then washed 3×15 min with the same buffer. The samples were then dehydrated in a graded ethanol series (50%, 70%, 80%, 90% and 100%) each for 10 min and in absolute water-free ethanol for 2×15 min. The samples were then embedded in LR-Gold diluted with ethanol: 1:3 for 14 hr, 1:1 for 10 hr, 3:1 for 14 hr and transferred to pure LR-Gold for 10 hr. Finally the samples were placed in pure LR-Gold in gelatinous capsules and polymerized for 24 hr.

The whole procedure was carried out at 4 °C and all solutions were filtered through a 0.2 μm sterile filter. Ultrathin sections (900–1000A) were attached to collodion coated nickel grids. The grids were washed in PBS and blocked for 30–45 min with a blocking solution (5% NS, Tween 20, 0.5% PBS) and then incubated overnight with anti PAA at 1:100 or 1:200 dilution and rinsed 6 times in PBS. The same was done with the preimmune serum. The grids were then incubated with the second-gold conjugated goat anti-rabbit IgG, 10 ng 1:25 in

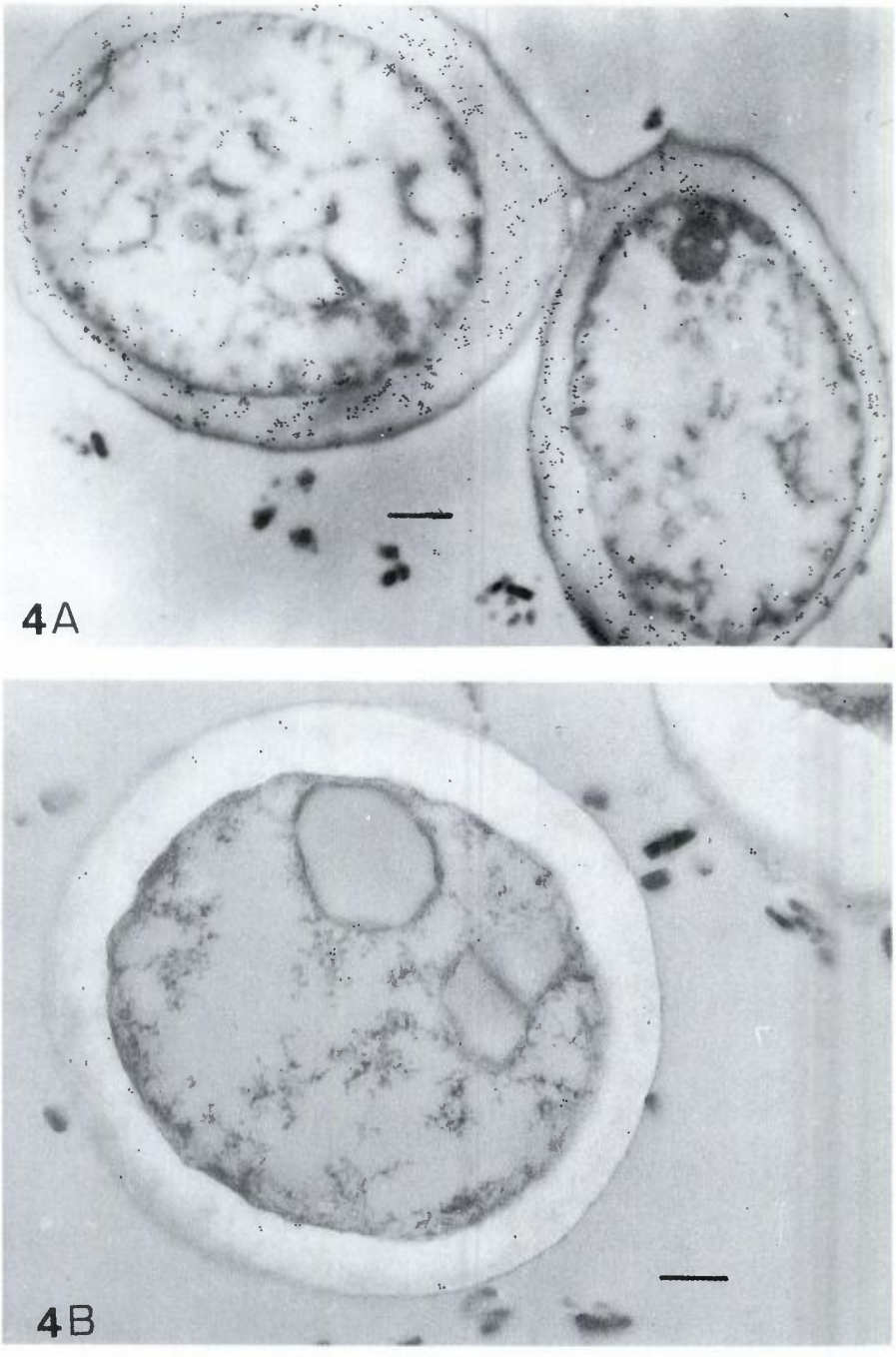

Figure 4. Electron microscope micrographs of sections in cephalodia of *P. aphthosa* stained with (A) antibodies against the lectin PAA1 or (B) the preimmune serum. Bar = 1 µm.

blocker for 1–2 hr at room temperature and washed 5× in PBS and 5× in dH_2O. Micrographs were recorded on a Phillips TEM-40 microscope at 100 kw.

3. Results

Agglutination assays indicated that the fraction eluted from the CMC column exhibited high agglutination activity which could be inhibited by lactose. Therefore, an affinity chromatography step on immobilized lactose was used for further purification.

Electrophoresis on SDS-PAGE of the two step purified extract of *P. aphthosa* revealed two closely migrating bands of approx. 20 kD (Fig. 2). After electrophoresis under non-denaturing conditions the silverstained native gel also showed two distinct bands PAA1 and PAA 2 (Fig. 3) indicating two different proteins. The two bands exerted from the native gel into PBS were tested for hemagglutination. The upper more pronounced band (Fig. 3, PAA1) showed agglutination activity.

Immunogold labelling using antibodies raised against the lectin PAA1 revealed a clear gold labelling of the fungal cell walls of fungal cells adjacent to the *Nostoc* cells in the cephalodia (Fig. 4 A). The control sections challenged with preimmune serum showed no immunogold labeling (Fig. 4 B).

4. Discussion

Lectins are very common in many organisms. In contrast to the wealth of knowledge regarding their structure and properties, we know rather little about the biological functions of these substances. However, the role of lectins as determinants of specificity in early cellular interactions involved in symbiosis and host-parasite associations is well established (Peumans and Van Damme, 1995; Hirsch, 1999; Lin et al., 2000).

We investigated the role of lectins in several lichen species, representatives of different taxonomic groups (Bubrick et al., 1981, 1985; Kardish et al., 1991; Lehr et al., 1995) questing the universality of this phenomenon.

In this study we show that a lectin produced by a vegetative fungal component of *P. aphthosa* has a similar function in selecting the compatible cephalodial cyanobacterium as lectins produced by germinating spores.

Cephalodia occur in about 520 lichen species and are usually located in distinctly delimited parts of the thallus (Jahns, 1988). The cephalodia of *P. aphthosa* are external structures on the upper surface of the thallus (Figs. 1 and 5) and contain *Nostoc* sp. A highly specific biorecognition process is involved in the acquisition of the cephalodial *Nostoc*. Even when various green algae or

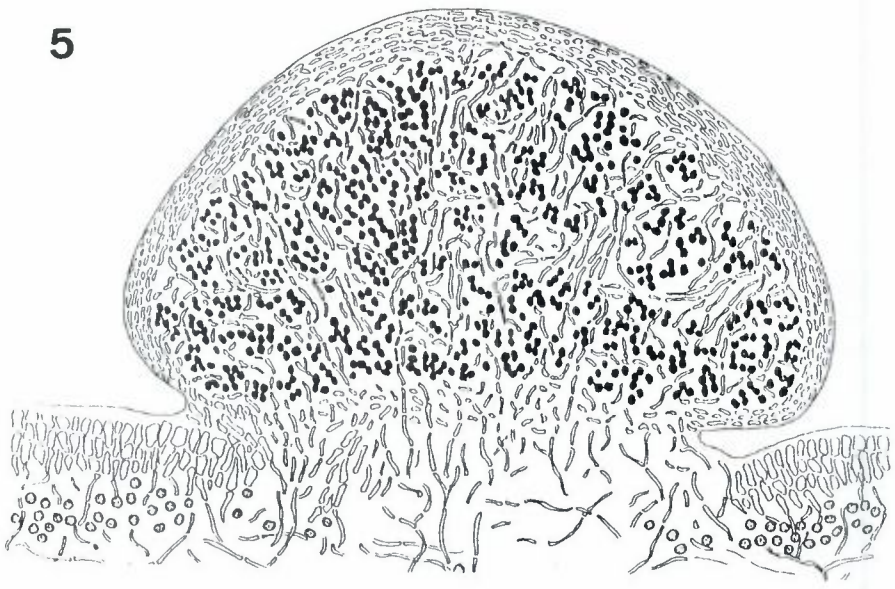

Figure 5. Schematic drawing of a cephalodium in section.

cyanobacteria land on the thallus only the compatible cyanobiont will be incorporated and the cephalodium formed. The selected cyanobiont becomes engolfed by the fungal hyphae. The cortex and algal layer (*Coccomyxa*) beneath the developing cephalodium dissociate and a direct contact between the cephalodium and the thallus interior is formed (Jahns, 1988) (Fig. 5). The acquisition of the *Nostoc* probably provides a nutritional advantage to the lichen.

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