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Lectin from the Lichen *Peltigera Membranacea* (Ach.) Nyl.: Characterization and Function

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Abstract

A specific lectin was isolated from the lichen *Peltigera membranacea* and purified to homogeneity by two chromatographic steps – carboxymethyl cellulose and hydroxyl appatite. This lectin (PMA) was found to be specifically inhibited by N-acetyl D-galactosamine. Fluorescamine-labelled PMA was used for microscopic visualization of its reaction with the homologous and some heterologous *Nostoc* cells. It was shown that the highly specific antibodies raised against PMA distinguished among several closely related species of *Peltigera*. These antibodies showed no affinity to the germ tubes from spores of *Peltigera praetextata* or *P. canina*. Partial sequence analysis of the PMA revealed no homology to any other known lectin, including NLA, the lectin of the cyanolichen *Nephroma laevigatum*.

1. Introduction

Symbiotic systems are stable associations of unrelated taxa. One of the most challenging problems facing symbiology is to determine the basis of mutual recognition and the level of specificity between the symbionts. Only in a few

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symbiotic associations is the molecular basis of selection understood, and in those cases it involves production of lectins that mediate between surface molecules of the symbionts.

In 1974 Bohlool and Schmidt proposed that "... an interaction between legume lectins and *Rhizobium* cells may account for the specificity expressed between rhizobia and host plants in the initiation of the nitrogen fixing symbiosis." Dazzo et al. (1978; 1988) showed that indeed a lectin produced by the legume root specifically recognizes molecules on the rhizobial surface for a compatible interaction.

In 1989 Diaz et al. introduced the pea lectin gene into white clover roots which resulted in nodulation of the clover roots by a *Rhizobium* strain specific for pea plants, further supporting the hypothesis of Bohlool and Schmidt. Other symbiotic systems have also been subjected to search for lectins that function as mediators.

We have shown (Bubrick et al., 1981; 1984; 1985; Kardish et al., 1991) that the fungal component (the mycobiont) of *Xanthoria parietina*, a green algal lichen, and the mycobiont of *Nephroma laevigatum*, a cyanobacterial lichen species each produce a different extracellular lectin. These lectins bind specifically to the compatible photobionts (*Trebouxia* sp. and *Nostoc* sp., respectively), and also distinguish between the symbiotic photobiont and the same after regaining its free-living growth form in culture. Lockhart et al. (1978), Petit (1982), and Petit et al. (1983) isolated a lectin from the lichen *Peltigera horizontalis* and another lectin from *P. canina*, both of which bind to the cultured homologous photobionts and to photobionts of some related *Peltigera* species. These authors also suggested that lectins may be involved in the recognition and initial interactions between compatible lichen symbionts.

In this work we characterize the purified lectin isolated from the lichen *Peltigera membranacea*, postulate its function and distinguish between a number of closely related *Peltigera* species by immunological criteria.

2. Materials and Methods

Organisms

(1) *Peltigera membranacea* (Ach.) Nyl. from Ullapool, Scotland, collected by J. Garty, October 1991. (2) *P. membranacea* from Rottensteiner Valley, Carintlia, Austria, collected by E. Stocker, June 1993. (3) *P. praetextata* (Sommerf.) Zopf, from Schwarzau, Pax-Valley, Lower Austria, collected by R. Türk, June 1993. (4) *P. praetextata* from Burg Rappottenstein, Waldviertel, Lower Austria, collected by J. Poelt and R. Türk, June 1994. (5) *P. praetextata*

from Chatzenschwanz, Riemenstalden, Canton Schwyz, Switzerland, collected by M. Galun, July 1993. (6) *P. praetextata* from between Höchi and Farlen, Riemenstalden, Canton Schwyz, Switzerland, collected by M. Galun, July 1993. (7) *P. praetextata* from Höchi, Riemenstalden, Canton Schwyz, Switzerland, collected by C. Scheidegger, May 1993. (8) Mixture of *P. praetextata* and *P. leucophlebia* (Nyl.) Gyeln., from Chatzenschwanz as above. (9) *P. canina* (L.) Willd., from Muothatal, Bödmerenwald near Mittst Weid, Canton Schwyz, Switzerland, collected by C. Scheidegger, October 1992. (10) *P. degeni* Gyeln., location and collection as in (6).

Determinations have been emended by O. Vitikainen. *Nostoc* spp. were isolated from *P. membranacea, Cycas revoluta* Thunb. (Botanical Garden, Tel-Aviv University), *Gunnera kaalensis* (Krajian) St. John (Mt. Kaala, Ohau, Hawaii), *Nephroma laevigatum* Ach. (Har Meron, Upper Galilee) and *Blasia pusilla* L. (Saarland, Germany). Isolation and culturing conditions were as described by Leizerovich et al. (1990).

Extraction of the lectin from P. membranacea (referred to as PMA – Peltigera membranacea agglutinin)

Five g (d.w.) of thallus material were homogenized with glass beads in a mortar and pestle with 50 ml phosphate buffered saline (PBS) (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, and 150 mM NaCl, pH 7.2) containing 3 mM dithiothreitol (DTT), 2 mM phenylmethylsulfonyl fluoride (PMSF), 15 mM sodium azide and 1% (v/v) Tween 80. The homogenate was stirred for 24 hr at 4°C and centrifuged at 12,000 g for 20 min. The supernatant was collected and stored at 4°C.

Lectin purification

The crude extract was dialyzed for 16 hr at 4°C against 10 mM sodium-phosphate buffer, pH 7.2. Purification of the lectin was carried out following the procedure of Petit et al. (1983) except that only two chromatographic steps, CMC (carboxymethyl-cellulose, Whatman) and HA (hydroxyl appatite) were used. Eight ml of the dialysate were applied to a CMC column (1.9 × 26 cm). The column was equilibrated with buffer as above and agglutinin-containing fractions were pooled.

The active fraction obtained from the CMC column was applied to a HA (hydroxyl appatite, Bio-Rad) column (1.9 × 13 cm). The column was equilibrated with 0.02 M potassium-phospate buffer, pH 6.9 and eluted with 50 ml of the same buffer followed by a linear gradient of 0.02 M to 0.5 M of this

buffer (200 ml). The agglutinin-containing fractions were pooled, dialyzed against ddH$_2$O for 16 hr at 4°C and concentrated 15-fold in a Speed-Vac apparatus (Savant Instruments, Framingdale, NY).

Agglutination assays

Agglutination assays were carried out in V-shaped microtiter plates, containing 50 µl of a 3% suspension of rabbit blood cells in PBS and 50 µl of crude extract or lectin solution (each serially diluted 2-fold). Agglutination was assessed visually after approximately 30 min at room temperature. One unit of activity is defined as the minimal amount of lectin required for hemagglutination under standard conditions. Throughout the purification procedures extracts were assayed for agglutination activity.

Protein assay

Protein concentration was measured by the method of Marder et al. (1986).

Sugar specificity of the lectin

Sugar specificity of the lectin was determined by scoring the inhibition of agglutination by a series of 14 sugars: D(+)glucose, 2-deoxy-D-glucose, N-acetyl-D-glucosamine, D(+)mannose, α-methyl-D-mannoside, D(–)arabinose, α-L-rhamnose, D(+)galactose, deoxy-D-galactose, D(+)galactosamine, N-acetyl-D-galactosamine, methyl-α-D-galactopyranoside, D-galacturonic acid, α-D(+)melibiose. Ten µl of purified lectin, after the HA column (25 µl/ml), were incubated for 30 min with 50 µl of each serially diluted carbohydrate solution (initial concentration 200 mM) in microtiter plates before adding the erythrocyte suspension and the result was scored after an additional 60 min at room temperature.

Selected physico-chemical treatments

The effect of salt concentrations, ethylenediaminotetraacetate (EDTA) and chaotropic agents on the agglutination activity was determined by incubating the crude extract (1.5 mg/ml protein) for 2 hr at room temperature in the presence of NaCl, EDTA-Na$_2$, urea and SDS, respectively. The samples were then dialyzed against PBS (pH 7.2) for 16 hr at 4°C and the agglutination activity tested.

The effect of temperature, enzyme treatments and polyvinylpyrrolidone (PVP) on agglutination activity were tested. Samples of crude preparations were heated for 10 min at 37°C, 42°C, 75°C, and 100°C, respectively, and changes of activity scored. Samples as above were incubated with trypsin or pronase (10 µl of 0.1 mg/ml solution in PBS were added to 300 µl crude extract) at 37°C and agglutination examined after 2 hr. The effect of insoluble PVP, treated to remove Cl⁻ (Loomis, 1974), was examined by adding PVP to the extraction buffer at final concentrations of 1% or 3%. The suspension was stirred for 16 hr at 4°C, then centrifuged at 12,000 g for 10 min and the clear supernatant used for testing.

The effect of pH (ranging from 3 to 10) on the lectin stability was tested. The pH of the samples was adjusted to pH 7 prior to agglutination activity determination.

Binding of fluorescamine-labelled lectin to photobionts

Homologous (fresh and cultured) and heterologous cultured *Nostoc* cells were incubated with fluorescamine-labelled lectin and binding was examined as described by Kardish et al. (1991).

Preparation of antiserum

Antiserum to the PMA was raised in female, approximately 3 month old "gelbsilber" rabbits according to Nilson and Larson (1990). The 22 kDa band was excised from the nitrocellulose membrane (see below) and 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 initial injection. Approximately 75 µg of protein in Incomplete Freund's Adjuvant were used for three subsequent booster injections at three week intervals. The rabbits were bled from the ear three weeks after each booster injection. The blood, after 3 hr in room temperature and overnight at 4°C was centrifuged (20,000 g for 30 min) and the supernatant was stored at –20°C until used.

Electrophoresis and immunoblotting

Electrophoresis and immunoblotting was performed as in Kardish et al. (1991) with slight modifications: the gel was of 12.5% polyacrylamide and was stained with Coomassie Brilliant Blue R250. Transfer was for 1 hr and 15 min in 12.5 mM ethanolamine/glycine buffer. The gel was then stained for

immunoprobing with a solution containing 0.1% Ponceau Red in 0.7% TCA and then left to dry. Following electrotransfer, the blots were blocked with 1% milk containing 0.02% sodium azide, incubated for 16 hr at room temperature with the anti-lectin diluted 1:2,000 in the blocking solution and shaken in a shaker bath at 100 strokes/min. After two washes with 0.1% Tween 20 in TBS (200 mM NaCl, 50 mM Tris-HCl, pH 7.4), followed by one wash with TBS, 10 min each, the blots were incubated for 1 hr with goat-anti-rabbit IgG coupled to alkaline phosphatase (Sigma) at a 1:30,000 dilution in TBS. Following two washes with TBS for 10 min each, the reaction product was visualized by treatment with 3.3 mg/10 ml of p-nitro-blue tetrazolium chloride and 1.7 mg/10 ml of 5-bromo-4 chloro-3 indol phosphate (both from Sigma) in development buffer (100 mM Tris-HCl, 100 mM NaCl, 2 mM $MgCl_2$, pH 9.5).

Molecular weight markers (Sigma SDS-7) were: bovine albumin 66 kDa, egg albumin 45 kDa, glyceraldehyde-3-phosphate dehydrogenase 36 kDa, carbonic anhydrate 29 kDa, trypsinogen 24 kDa, soybean trypsin inhibitor 20.1 kDa, alpha lactoalbumin 14.2 kDa.

Extraction of total protein

One g of thallus of each of the *Peltigera* collections (see above) were homogenized using a mortar and pestle in 10 ml PBS in the presence of glass beads. The slurry was stirred for 16 hr, centrifuged for 5 min at 12,000 g and the supernatant collected and stored at 4°C until used. Protein concentration was the same in all samples (1.5–2.0 µg/µl). Eight µl of each extract were loaded on a 12.5% acrylamide gel and separated as described above. After transfer to the nitrocellulose membrane blotted proteins were incubated with the *P. membranacea* antibody, which recognized a ladder of bands.

Preparation for microsequencing

The dried lectin (75 µg) was dissolved in 100 µl of 70% formic acid. Cyanogen-bromide (CNBr) (15 mg) was dissolved in 100 µl of 70% formic acid and diluted 100-fold by adding 1 µl to 100 µl of the lectin solution by stirring under N_2-saturated atmosphere, for 20 sec. The sample was then kept in the dark for 24 hr, diluted 10-fold with ddH_2O and dried overnight in a dessicator under vacuum in a fume hood. The sample was then twice dissolved with water and dried in a Speed-Vac apparatus. Finally it was dissolved in a minimal amount of water and sample buffer. The pH was adjusted with 2 M Tris-HCl to pH 7. The sample to be sequenced was prepared by running approximately 35 µg/lane of total protein on SDS-PAGE. The prepared polyacrylamide gel

refrigerated for 48 hr to ensure complete polymerization. Thioglycolate (11.4 mg/l) was added to the running buffer. The protein was electrotransfered to a ProBlott™ membrane in a CAPS (3-cyclohexylamino-1-propane-sulfonic acid) buffer containing 5 mM CAPS and 10% methanol for 3 hr at 75 V. Following electrotransfer the ProBlott™ was rapidly stained (30–60 sec) in a 0.1% Amido Black solution containing 40% methanol (HPLC grade) and 1% acetic acid. Destaining was in ddH$_2$O. The major largest fragment was subjected to N-terminal sequencing. This material was first cleaved by trypsin and the tryptic peptides were isolated on a HPLC RP-18 column. The major peaks were tested by mass spectrometry and three of the peptides were analyzed in an automated peptide sequencer.

3. Results

A specific lectin (PMA) from *P. membranacea* was purified to homogeneity by two chromatographic steps and similarly to the results of Petit et al. (1983), the active material eluted at the void volume of the CMC column. The agglutinating fraction eluted from the HA column revealed a single band by SDS-polyacrylamide gel electrophoresis with the apparent molecular mass of 22 kDa (Fig. 1). This resembles the molecular mass of the lectin isolated by Petit et al. (1983) from "*Peltigera canina* var *canina*".

The agglutinating activity of the purified lectin was 10,000 units/mg protein. Agglutinating activity was completely inhibited by N-acetyl-D-galactosamine and partially inhibited by the related sugars D(+)galactose and methyl-α-D-galactopyranoside. Partial inhibition was also observed with D(+)melobiose. None of the other sugars tested inhibited the lectin.

Agglutination was completely inhibited by treatment at 75°C and 100°C, while lower temperatures had no effect. Other treatments had either a partial inhibition effect or none at all (Table 1). Activity was maintained at pH 4 to 7.

The lectin labelled with fluorescamine reacted with the homologous and some heterologous *Nostocs* that originated from other symbiotic systems (Table 2). The lectin bound to the symbiotic *Nostoc* cells of *P. membranacea* and to hormogonia of the *P. membranacea Nostoc* grown in culture (Fig. 2a and b). Cultured *Nostoc* isolated from *Cycas revoluta* also reacted positively with the lectin, whereas *Nostoc* isolated from *Nephroma laevigatum*, from *Gunnera kaalensis* and from *Blasia pusilla* showed no reaction (Table 2).

As we had no success in germinating the spores of *P. membranacea*, we could not examine whether the lectin is a product of the fungal partner, as in *Xanthoria parietina* (Bubrick et al., 1981) and in *N. laevigatum* (Kardish et
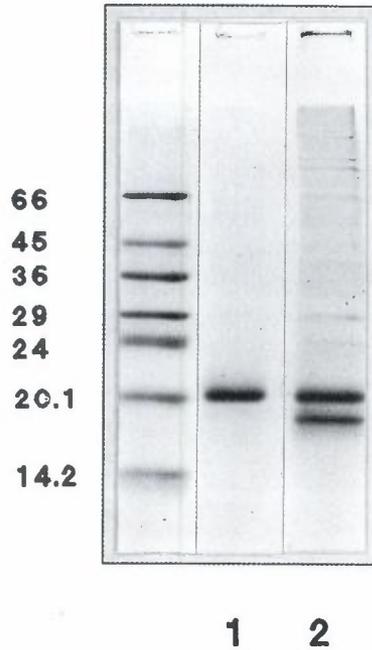
Figure 1. SDS-PAGE of purified (1) and crude (2) fractions isolated from *Peltigera membranacea*, 5 μg of the purified and 20 μg of the crude extract were applied to the slots. Positions of molecular mass markers are indicated at the left (size given in kilodaltons).
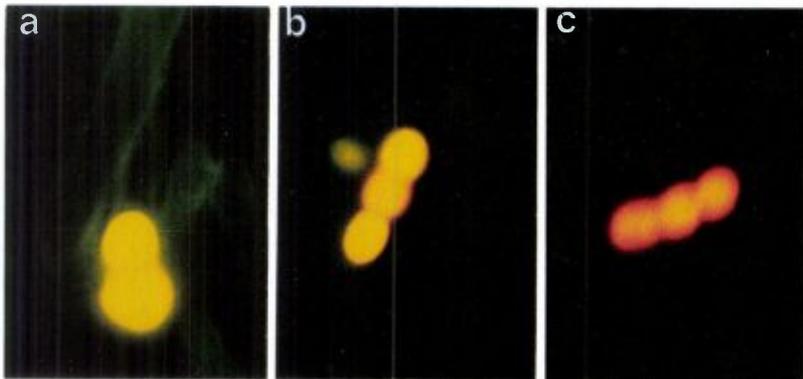

Figure 2. Fluorescamine-labelled lectin staining: (a) symbiotic (freshly isolated) *Nostoc* of *Peltigera membranacea* – positive reaction; (b) cultured *Nostoc* (hormogonia) of *P. membranacea* – positive reaction; (c) control, homologous *Nostoc* (without fluorescamine-lalbelled lectin) – no reaction.

Table 1.   Effect of various treatments on PMA agglutination activity (crude extract).

Treatment	% of activity
37°C	100
42°C	100
75°C	0
100°C	0
1% PVP (polyvinylpyrrolidone)	100
3% PVP (polyvinylpyrrolidone)	100
Trypsin 3%	50
Pronase 3%	50
0.5 M NaCl	100
0.66 M NaCl	100
1 M NaCl	50
0.1% SDS	25
6 M urea	100
6 M urea + 10 mM β ME (mercaptoethanol)	100
6 M urea + 5 mM DTT (dithiothreitol)	75
EDTA-Na$_2$ 3%	100

Table 2.   Binding of PMA to *Nostoc* spp.

Nostoc from	Binding
Peltigera membranacea – cultured	+
Peltigera membranacea – symbiotic	+
Cycas revoluta	+
Gunnera kaalensis	−
Nephroma laevigatum	−
Blasia pusilla	−

al., 1991).   The antibodies raised against the lectin from *P. membranacea* showed no affinity to the germ tubes of *P. praetextata* spores nor to those in *P. canina*.

The protein extract of *P. canina* after transfer to the nitrocellulose membrane did not react with αPMA and is therefore shown on the blot prior to the immuno-reaction (Fig. 3).   Western blotting using αPMA revealed that each of the *Peltigera* species examined had a different pattern of proteins (Fig. 3). None of the other species showed a band that corresponded to PMA.

Figure 3. Western blotting. Total protein was extracted from the various lichens and 8 µl of total protein of each was fractionated on SDS-PAGE, electroblotted onto nitrocellulose and immunochemically detected using PMA antibodies. Lanes 1 and 2: *Peltigera membranacea*; lanes 3–7: *P. praetextata*; lane 8: mixture of *P. praetextata* and *P. leucophlebia*; lane 9: *P. canina*; lane 10: *P. degeni*. The protein extract of *P. canina* did not react with the antiserum used (lane 9); lane 9* shows the protein extract on the blot prior to the immuno-reaction. Positions of molecular mass markers (kDa) are indicated on the left of the blot.

The sequences of the three peptides analyzed are shown in Table 3. Sequence homology search revealed no homology of PMA with the lectin of the cyanolichen *Nephroma laevigatum* (unpublished data) nor to any other lectin.

Table 3.   Amino acid sequences of peptides derived from PMA by tryptic digestion.

Peptide	Position																						
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
I	K/R	A	V	Y	S	N	P	V	V	I	Q	V	F	D	N		G	T	A	A	G	P	P	T
II	K/R	S	N	Y	H	N	I	Y	V	F/I	D	V	I/F										
III	K/R	I	Y	T	F	D	K	R															

4. Discussion

The extraction and purification procedures we employed were the same as by Petit et al. (1983) for the lectin extracted from "*Peltigera canina* var *canina.*" The molecular mass of lectin was similar in both cases. Since the difficulty in correct identification of the *Peltigera*s in this group, we assume that we both used the same lichen species. As the identification of our specimens was in part verified and in part the species determined by O. Vitikainen (who is a specialist in *Peltigera*) we believe that the species used for the lectin extraction was in both cases *Peltigera membranacea*.

The ladder of bands on the Western blot (Fig. 3) helps to distinguish between the different, but closely related, species, a distinction which is in accord with the determinations (by conventional means) by Vitikainen. None of the other species examined showed a band on the Western blot that corresponded with the *P. membranacea* lectin. Specificity is also suggested by the observation that none of the proteins extracted from *P. canina* (Fig. 3, lane 9) reacted with αPMA.

Although it was not the initial intention of this study, the outcome suggests that immunological criteria might be useful for the distinction between closely related species, when other means do not result in unequivocal identification.

In contrast to the report by Petit et al. (1983), that the lectin did not bind to the freshly isolated photobiont, we found binding to fluorescamine-labelled PMA both to the symbiotic and the cultured homologous *Nostoc* (Fig. 2). This differs also from the binding pattern of ABP (the lectin of *Xanthoria parietina*) and of NLA (the lectin of *Nephroma laevigatum*), which distinguish between the symbiotic and the cultured homologous photobionts (Bubrick and Galun, 1980; Kardish et al., 1991). This is, however, not surprising since lichenization, that causes dramatic modifications particularly to the cyanobiont of *N. laevigatum* (Kardish et al., 1989), has a much lesser effect on the cyanobiont of *P. membranacea*. The latter retains much of its free-living growth form when integrated in the lichen thallus (not shown). It appears that the cultured cyanobiont cells of *Cycas revoluta* share some common surface determinants with the *P. membranacea* cyanobiont (Table 2). Some common molecules on the surface of heterologous cyanobionts have been observed previously as well (Kardish et al., 1991).

We have no direct evidence, as we had in previous tests (Bubrick et al., 1981; Kardish et al., 1991), that PMA is an extracellular product of the fungal partner of *P. membranacea* that recognizes receptors on the compatible photobiont, since we were not able to germinate the spores of *P. membranacea*.

However, the present results support the notion that involvement of lectins will prove to be widespread and that they, despite the lack of sequence

homology, may have similar functions in the recognition process between symbionts. Similarly to the proteins that are presumably involved in the recognition systems that plants have developed for detecting pathogens and triggering the defense response in incompatible interactions (Templeton et al., 1994).

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