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# Proteins From the Lichen *Xanthoria parietina* (L.)Th.Fr. which Bind to Phycobiont Cell Walls

## Isolation and Partial Purification of an Algal-Binding Protein

P. BUBRICK[1*], A. FRENSDORFF[2] and M. GALUN[1†]

[1] Department of Botany and [2] Department of Microbiology, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel. Tel. 972-3-420163; Telex 342171 VERSY IL.

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## Abstract

An algal-binding protein (ABP) has been isolated from the lichen, *Xanthoria parietina* (L.)Th.Fr. ABP was partially purified and had an estimated molecular weight of 12-13,000 dalton. Both ConA and RCA bound to native ABP. The interaction of [$^{125}$I]-ABP and phycobiont cells was examined in non-competitive and competitive binding assays. Cells apparently possessed high capacity binding sites for [$^{125}$I]-ABP. In terms of binding, [$^{125}$I]-ABP could discriminate between various cultured phycobionts. Preliminary evidence for the polysaccharide nature of the algal binding sites is presented. A possible role of ABP during the initial stages of biont resynthesis is discussed.

Key words: Lichen, phycobiont, algal-binding protein, resynthesis of lichen symbionts

## 1. Introduction

A lichen is an association between an alga and/or cyanobacterium (phycobiont) and a fungus (mycobiont). The range of possible combinations between these bionts is restricted; most lichen fungi can associate with only one or a few types of phycobionts. Evidence to support this view comes from two main sources: the isolation and identification of phycobionts, and the study of lichen resynthesis *in situ* and *in vitro* (summarized in Bubrick et al., 1985; Galun and Bubrick, 1984). Such a restricted range of biont combinations suggests that pycobionts may possess a mechanism(s) for the discrimination between potential phycobionts and other phycobionts or free-living algae.

* Present Address: Department of Plant Pathology, University of California at Riverside, Riverside, CA 92521, USA. Tel. (714) 787-4134.

† Author to whom offprint requests should be directed.

Limited progress has been made in the detection of possible molecular mechanisms which underlie this discriminatory ability. Lockhart et al. (1978) first suggested that lectins may play a role in this process in the lichen genus *Peltigera* (symbiotic with *Nostoc*). They found that protein extracts from *P. polydactyla* contained a lectin which bound to the *in vitro* cultured *Nostoc* biont from this lichen. In terms of binding, the lectin discriminated between *Nostoc* from *P. polydactyla* and a number of other symbiotic and free-living cyanobacteria. Similar results were obtained with crude protein extracts containing lectin (Petit, 1982) or with purified lectin (Petit et al., 1983) from other *Peltigera* species. It has been proposed that such lectins may play a role in the "recognition", or initial interactions between compatible lichen bionts (Petit et al., 1983).

Crude protein extracts from the lichen *Xanthoria parietina* also contained a component(s) which bound to cells of the normal cultured phycobiont from this lichen (Bubrick and Galun, 1980; Hersoug, 1983). As with lectins, this component(s) could also discriminate between the cultured *X. parietina* phycobiont and cultured phycobionts from other lichens.

In this study we report the isolation and partial purification of an algal-binding protein (ABP)* from *X. parietina*. Characteristics of the binding of ABP to phycobiont cells were also examined. A possible role for ABP during the initial stages of the reassociation between separated bionts is discussed. Part of the results given here have been presented in conference proceedings (Galun et al., 1984).

## 2. Materials and Methods

### 2.1 *Reagents*

Reagents were obtained from the following sources: Amersham International Ltd.: carrier-free [$^{125}$I]Na; BDH Chemicals Ltd.: BSA, DTT, SDS, trypsin (beef pancreatic); E-Y Laboratories Inc.: FITC-ConA, -DBA, -PNA, -RCA, -SBA, -UEA; Pierce Chemical Co.: Iodogen; Sigma Chemical Co.: PMSF, CBB-R250, chitotriose, horseradish peroxidase, ovalbumin, protease (pancreatic crude type I), Shiff's reagent.

### 2.2 *Organisms and growth conditions*

The lichens used in this study were collected locally in Israel. The isolation, purification and maintenance of lichen phycobionts have been described in Bubrick

* Abbreviations: ABP = algal-binding protein; AS = ammonium sulfate; BSA = bovine serum albumin; CBB-R250 = coomassie brilliant blue R250; DTT = dithiothreitol; FITC = fluorescein isothiocyanate; PBS = phosphate buffered saline; PBS-BSA = PBS buffered BSA (1% w/v); PAGE = polyacrylamide gel electrophoresis; PMSF = phenylmethylsulphonyl fluoride; RT = room temperature; SDS = sodium dodecyl sulfate.

and Galun (1980).

## 2.3 *Extraction and partial purification of ABP*

Thalli of *X. parietina* (5-10 g fresh wt.) were disrupted by hand in a mortar containing PBS ($KH_2PO_4$, 1.5 mM; $Na_2HPO_4$, 3.0 mM; KCl, 2.7 mM; $MgSO_4$, 0.4 mM; NaCl, 137.9 mM; pH 7.4); tissue was extracted with PBS (50 ml) containing PMSF (5.0 mM), DTT (2.32 mM), and sodium azide (15.0 mM) for 18 hr at 4°C. Tissue was pelleted (30,000 × g, 45 min), re-extracted with PBS (2 hr, 4°C), centrifuged, and the two supernatants combined. The supernatant was adjusted to 40% saturation with AS. After incubation (2 hr, 4°C), the solution was centrifuged (30,000 × g, 45 min), the pellet discarded, and the supernatant adjusted to 60% saturation with AS. After incubation and centrifugation as described, the supernatant was discarded, the pellet dissolved in a small volume (2-4 ml) of PBS, and dialized (3,500 d cut-off) against several changes of distilled water for 24 hr at 4°C. Following diaylsis, the contents of the bag were microfuged, the supernatant discarded, and the pellet redissolved in PBS. Soluble crude protein was stored in small aliquots at −20°C.

PAGE was employed for the detection and partial purification of ABP. Homogeneous, discontinuous PAGE was performed according to Laemmli (1970) on vertical slabs (15 cm × 18 cm × 2 mm). For ABP detections, aliquots of crude protein were extensively absorbed with cells of the cultured *X. parietina* phycobiont, and then compared to unabsorbed protein aliquots by PAGE. Crude protein (50-200 $\mu$g in 100-200 $\mu$l PBS) was added to a pellet of cultured cells ($10^5$-$10^6$), the cells suspended, and the mixture incubated (1 hr, RT) with frequent manual mixing. This was repeated three times, each time using new cultured cells. Aliquots of unabsorbed and absorbed crude protein were lyophilized, and stored at -20°C.

For PAGE, aliquots were dissolved in tris (0.125 M, pH 6.8) containing glycerol (10% w/v) (non-denaturing) or in tris containing glycerol, SDS (3% w/v) and DTT (1% w/v) with heating for 20 min at 70°C (denaturing). Protein was electrophoresed (100 v) at 5-8°C (non-denaturing) or RT (denaturing). Gels were stained for protein with CBB-R250 or for polysaccharide according to Zacharius et al. (1969).

Protein was also visualized by autoradiography of iodinated protein (see below). Following destaining, gels were dried onto Whatman 3 MM paper with a vacuum drier. Film (Agfa Curix x-ray film) was exposed at -70°C with an intensifying screen (Dupont cornex lighting – plus XL), and developed according to the manufacturer's recommendations.

Once localized on gels, ABP was isolated by elution from non-denaturing PAGE gels. Acrylamide regions (Rf 0.95-1.00) were cut out, and ABP eluted by diffusion into PBS. Eluates from several gels were combined, dialized (3,500 d cut-off) against PBS, and soluble protein stored at −20°C.

### 2.4 *Binding of FITC-lectins to ABP*

Dots of nitrocellulose (ca 5 mm diameter) were cut with a hole puncher and placed on a microscope slide. ABP (10 $\mu$g in 10 $\mu$l PBS) was added to the dot and allowed to dry. Dots were then immersed in PBS buffered BSA (5% w/v, 200 $\mu$l) and incubated for 1 hr at RT. Dots were washed with PBS and arranged on a microscope slide (10 dots per slide). FITC-lectin (1 $\mu$g/$\mu$g, 50 $\mu$l) was added to a dot and incubated (1 hr, RT) in a humid chamber. Dots were extensively washed with PBS (6 $\times$ 10 min, 300 $\mu$l), placed on slides and examined in a fluorescence microscope using low magnification and epi-illumination optics (BG-12 excitation filter, No. 50 barrier filter).

Competitive inhibition of FITC-lectin binding was studied by preincubating FITC-lectin (double concentration) and an appropriate sugar competitor (final concentration of sugar 0.1 M) for 30 min at RT and then adding the solution to the dot.

Controls included nitrocellulose dots only, dots with immobilized ABP and coated with BSA, and dots with immobilized BSA and incubated with FITC-lectins (all such controls were negative). Positive controls were done with horseradish peroxidase and ovalbumin. The FITC-lectins used are summarized in Table 1.

### 2.5 *Binding of ABP to cultured phycobiont cells*

The binding of ABP to phycobiont cells was studied using the probe, [$^{125}$I]-ABP. ABP was radio-iodinated with Iodogen basically as described by Markwell and Fox (1978) using ABP (100 $\mu$g/100$\mu$l PBS), [$^{125}$I]Na (1 m Ci), Iodogen (20 $\mu$g), and a two minute reaction time at RT. Unbound $^{125}$I was removed by dialysis (3,500 d cut-off) against several changes of PBS. [$^{125}$I]-ABP was diluted in PBS-BSA and stored in small aliquots at -20°C.

Whole algal cells ($10^4$) were immobilized in wells of PVC microtiter plates as previously described (Bubrick et al., 1982). For non-competitive binding assays, [$^{125}$I]-ABP (in 25 $\mu$l PBS-BSA) was added to wells and incubated for 1 hr at RT. Unbound ligand was aspirated and wells washed (4$\times$) with PBS. For competitive assays, cells were first incubated (1 hr, RT) with unlabeled ABP (in 25 $\mu$l PBS-BSA) and washed (4$\times$) with PBS. [$^{125}$I]-ABP (1 $\mu$g in 25 $\mu$l PBS-BSA) was then added to the cells, and treated as described for non-competitive assays. After assays were completed, plates were air dried, wells removed and counted in a gamma counter. All tests were done in triplicate; assays were repeated at least three times and with more than two batches of labeled ABP.

For some experiments, phycobiont cells were modified prior to incubation with [$^{125}$I]-ABP. All such tests were done in polypropylene centrifuge tubes (1.5 ml) containing a pellet of $10^4$ cells; all incubation steps were done on an end-over-end mixer. The treatments used are tabulated below:

1. EDTA, Urea, SDS: Phosphate ($K_2HPO_4$, 0.2 M; pH 8.5) buffered EDTA (0.1 M), PBS buffered urea (8.0 M) or PBS buffered SDS (1% w/v) was added to the cells, cells suspended, and incubated for 1 hr at RT. Cells were washed (4×) with PBS.
2. Heat: Cells, dispersed in PBS, were immersed in a boiling water bath for 10 min; after cooling, cells were washed (4×) with PBS.
3. Protease: PBS (200 $\mu$l) was added to the pellet and cells dispersed. Non-specific protease (0.2 mg) was added to the tube and incubated for 1 hr at RT. Cells were washed (4×) with PBS-BSA.
4. Trypsin: PBS (200 $\mu$l) was added to the pellet and cells dispersed. Trypsin (0.4 mg) was added to the tube and incubated for 2 hr at RT. PMSF (5 mM) was added to the tube, and cells washed (4×) with PBS.
5. Periodate: Sodium acetate (0.1 M; pH 5.0) buffered sodium metaperiodate (50 mM) was added to the pellet, the cells dispersed and incubated for 4 hr at RT in the dark. Ethylene glycol (1% w/v) was added to the tubes, incubated 30 min at RT, and cells washed (4×) with PBS.

Following treatments and washings, cells were dispersed in PBS (50 $\mu$l) to which [$^{125}$I]-ABP (50 $\mu$l in PBS-BSA) was added and incubated for 1 hr at RT with frequent manual mixing. Cells were washed (4×) with PBS and counted in a gamma counter. All tests were done in duplicate and repeated at least once.

### 2.6 *Other methods*

Protein concentrations were estimated according to Lowry et al. (1951), or according to Castell et al. (1979) using BSA as a standard.

## 3. Results and Discussion

The presence of an algal-binding protein (ABP) in crude protein extracts from *X. parietina* was detected in a novel fashion. Crude protein was extensively absorbed with cultured phycobiont cells, and then compared to unabsorbed protein by PAGE. It was reasoned that the binding component would be removed by absorption and detected as an electrophoretic band missing from absorbed protein. Such a comparison is shown in Fig. 1. One protein band (Fig. 1 "a") was consistently reduced by absorption; a second band (Fig.1 "b") was inconsistently affected. Thus, while recognizing the possibility of more than one binding component, band "a" (Fig. 1, lane 1) was chosen for further study and referred to hereafter as ABP.

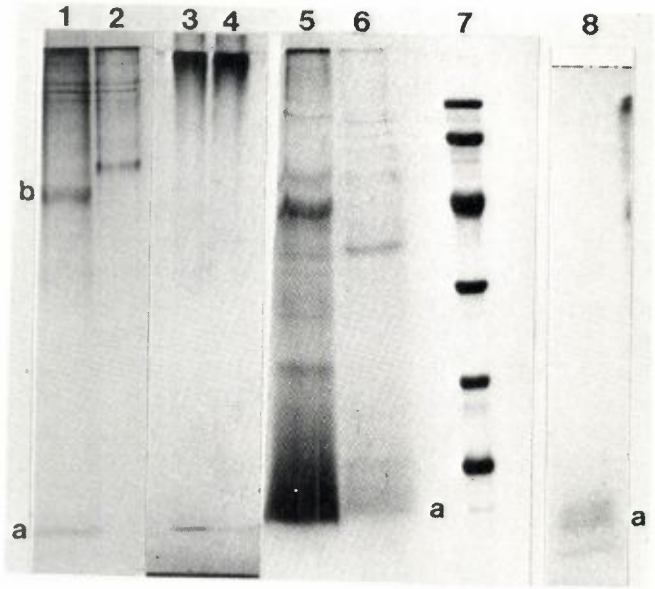


**Figure 1**. Detection and partial purification of ABP by PAGE. Protein bands labeled "a" and "b" are discussed in text. Band "a" corresponds to ABP. Lanes 1-4 non-denaturing PAGE; lanes 5-8 denaturing PAGE. Lanes 1,2,5-7 stained for protein; 3,4 stained for polysaccharide; 8 autoradiograph of [$^{125}$I]-ABP. Lanes 1,3: unabsorbed ABP (30 $\mu$g); 2,4: absorbed ABP (<30 $\mu$g); 5: unabsorbed ABP (20 $\mu$g); 6: absorbed ABP (< 20 $\mu$g); 7: molecular weight standards (Pharmacia) (from top) phosphorylase-b (94 kd), BSA (67 kd), ovalbumin (43 kd), carbonic anhydrase (30 kd), trypsin inhibitor (20.1 kd), $\alpha$-lactalbumin (14.4 kd); 8: autoradiograph of partially purified ABP, eluted from non-denaturing PAGE gels, radioiodinated and electrophoresed under denaturing conditions.

ABP was partially purified by preparative electrophoresis. Following elution from non-denaturing gels, ABP was radioiodinated and subject to SDS-PAGE (Fig. 1, lane 8). Gel autoradiographs showed that [$^{125}$I]-ABP consisted of two closely migrating bands in the molecular weight range of 12-13,000 dalton.

Sugar molecules associated with non-denatured ABP were detected using FITC-lectins (Table 1). Both FITC-ConA and -RCA bound to ABP; binding was competitively inhibited by $\alpha$-methyl-D-mannoside and $\alpha$-D-galactose respectively. FITC-WGA also bound to ABP but binding was not inhibited by chitotriose or ovalbumin.

The interaction of [$^{125}$I]-ABP with cultured phycobiont cells was studied in

**Table 1**. Interaction of FITC-lectins with ABP[a]

| | FITC- | | | |
|---|---|---|---|---|
| | ConA[b] | WGA | RCA | PNA |
| Horseradish peroxidase | + | − | − | − |
| Ovalbumin | − | + | − | − |
| ABP | + | +[c] | + | − |

[a] 1 $\mu$g glycoprotein/nitrocellulose dot.

[b] Abbreviations: ConA = concanavalin A; WGA = wheat germ agglutinin; RCA = lectin from castor bean; PNA = lectin from peanut.

[c] Binding not inhibited by chitotriose or ovalbumin.

**Table 2**. Binding of [$^{125}$I]-ABP to the cultured phycobiont from *X. parietina*[a,b]

| [$^{125}$I]-ABP added (ng) | [$^{125}$I]-ABP bound (ng) | B/F ($\times 10^{-2}$) |
|---|---|---|
| 1610.7 ± 55.9 | 23.3 ± 1.7 | 1.5 |
| 855.3 ± 22.7 | 14.0 ± 0.3 | 1.7 |
| 461.0 ± 0.0 | 6.2 ± 0.3 | 1.4 |
| 225.3 ± 7.8 | 2.9 ± 0.4 | 1.3 |
| 114.6 ± 3.1 | 1.9 ± 0.4 | 1.7 |
| 61.4 ± 1.2 | 0.9 ± 0.1 | 1.5 |

[a] $10^4$ cells/test. Specific activity of [$^{125}$I]-ABP was 629 cpm/ng.

[b] Non-specific binding of [$^{125}$I]-ABP to microtiter wells has been subtracted.

[c] B/F = ratio of bound [$^{125}$I]-ABP to free [$^{125}$I]-ABP.

non-competitive and competitive binding assays. In non-competitive assays (Table 2) the binding of [$^{125}$I]-ABP to cells was linear over a wide range of ligand concentrations and saturation of binding sites was not achieved. When data were plotted according to Scatchard (1949), a near horizontal line was obtained (not shown). Competitive binding assays (Table 3) demonstrated that ABP competed with [$^{125}$I]-ABP for available binding sites on phycobiont cells. However, relatively large concentrations of ABP were required before significant inhibition was observed.

The results from Tables 2 and 3 suggest that phycobiont cells can bind significant quantities of ABP. The nature of the binding site(s) is unclear although available data do not suggest the presence of a discrete cellular "receptor" for ABP. Such results might be interpreted as indicating that the binding of ABP to phycobiont cells is a "non-specific" phenomenon. However, [$^{125}$I]-ABP bound in a quantitatively discriminatory manner to the cultured *X. parietina* phycobiont

**Table 3.** Competitive inhibition of [$^{125}$I]-ABP binding to the cultures *X. parietina* phycobiont by ABP[a,b]

| Unlabeled ABP added (ng) | [$^{125}$I]-ABP bound (%)[c] |
|---|---|
| 0 | 100 |
| 200 | 92 |
| 500 | 87 |
| 1000 | 71 |
| 10000 | 52 |

[a] $10^4$ cells/test. Specific activity of [$^{125}$I]-ABP was 3960 cpm/ng. Input of [$^{125}$I]-ABP was 1000 ng/test.

[b] As in Table 2.

[c] Calculated as [$^{125}$I]-ABP bound after preincubation of cells with ABP/[$^{125}$I]-ABP bound in the absence of ABP (= 13 ng) × 100.

**Table 4.** Binding of [$^{125}$I]-ABP to different cultured phycobionts[a,b]

| Cultured phycobiont from | [$^{125}$I]-ABP bound | |
|---|---|---|
| | ng | %[c] |
| *Xanthoria parietina* | 5.3 | 100.0 |
| *X. steineri* | 0.5 | 9.4 |
| *Physconia pulverulenta* | 1.6 | 30.2 |
| *Ramalina pollinaria* | 0.3 | 5.7 |
| *Peltigera aphthosa* | 0.2 | 3.8 |

[a] $10^4$ cells/test except for *Coccomyxa* phycobiont from *P. aphthosa* ($10^5$/test). All phycobiont cells except *Coccomyxa* had roughly the same volume. Specific activity of [$^{125}$I]-ABP was 820 cpm/ng. Input of [$^{125}$I]-ABP was 250 ng/test.

[b] As in Table 2.

[c] Calculated as cpm bound to heterologous phycobiont/cpm bound to *X. parietina* phycobiont × 100.

(Table 4). This clearly suggests that there are binding sites on this phycobiont which are not expressed/available on other cultured phycobionts. Thus, [$^{125}$I]-ABP binding is not viewed as a purely "non-specific" phenomenon.

The nature of the ABP binding site(s) was further examined by exposing the cultured *X. parietina* phycobiont to a number of physical and chemical treatments

**Table 5**. Modification of the cultured phycobiont from *X. parietina* and its effect on [$^{125}$I]-ABP binding[a]

| | Treatment[b] | [$^{125}$I]-ABP bound after treatment (%)[c] |
|---|---|---|
| EDTA | (0.1 M, 1 hr) | 100 |
| Urea | (8.0 M, 1 hr) | 100 |
| Heat | (100°C, 10 min) | 100 |
| SDS | (1% w/v, 1 hr) | 976 |
| Protease | (0.1% w/v, 1 hr) | 354 |
| Trypsin | (0.2% w/v, 2 hr) | 74 |
| Periodate | (30 mM, 4 hr) | 80 |

[a] $10^5$ cells/test. Input of [$^{125}$I]-ABP was 500 ng/test.

[b] All reagents buffered in PBS except periodate (0.1 M sodium acetate, pH 5.0).

[c] Calculated as [$^{125}$I]-ABP bound after treatment/[$^{125}$I]-ABP bound after PBS treatment or inhibited reaction mixture treatment × 100.

and testing for binding of [$^{125}$I]-ABP to modified cells (Table 5). Exposure of cells to EDTA or urea had no effect on binding, suggesting that binding sites are an integral part of the cell wall. Treatment of cells with SDS or non-specific protease greatly increased [$^{125}$I]-ABP binding, suggesting that these reagents exposed new binding sites. Cells treated with either trypsin or periodate bound less [$^{125}$I]-ABP. This taken with the observation that heat did not effect [$^{125}$I]-ABP binding, implied that the binding site(s) was polysaccharide in nature and possibly anchored in the wall by a trypsin sensitive bond. The relationship between [$^{125}$I]-ABP binding following protease or trypsin treatment is not presently understood.

These results, as well as previous studies, suggest a possible role for ABP during the early stages of symbiont interaction. The potential for *in situ* resynthesis between separated *X. parietina* bionts probably exists (Bubrick et al., 1984). The role of ABP in this process is viewed as the binding and immobilization of potential phycobiont cells onto mycobiont hyphae. Such an hypothesis takes into account the apparent high binding capacity of cells for ABP (Tables 2, 3) as well as the discriminatory binding properties of ABP (Table 4). The apparent lack of an algal cell wall "receptor" for ABP does not detract from this proposed role, since highly selective interactions between different cell types can arise from apparently "non-receptor"-ligand interactions (Calleja et al., 1981; Rao et al., 1982; Young and Kauss, 1984).

Following immobilization of cells on hyphae, envelopment of algal cells takes

place (Bubrick et al., 1985). It is not presently known whether envelopment is a direct response to ABP binding or is elicited by a distinct mechanism. In any case, the binding of ABP to potential phycobiont cells may represent one of the first steps in the resynthesis of isolate *X. parietina* bionts.

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