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Binding of a Labelled Lectin from the Lichen *Xanthoria parietina* to its own Phycobiont and Analysis of its Enzymatic Activity

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With 4 Figures

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Summary

MOLINA M. C., VICENTE C., PEDROSA M. M. & MUNIZ C. 1996. Binding of a Labelled Lectin from the Lichen *Xanthoria parietina* to its own Phycobiont and Analysis of its Enzymatic Activity. – Phyton (Horn, Austria) 36 (1): 145–158, 4 figures. – English with German summary.

A partially purified algal-binding protein (ABP) has been purified from *Xanthoria parietina* thalli. This algal-binding protein behaves as a phytohaemagglutinin against human erythrocytes and contains arginase activity. About 30% of ABP is labelled by using saturating concentrations of ferritin or fluorescein, by ramdomized chemical interaction. Labelled ABP binds to 24%–30% of *Xanthoria* phycobionts when they contain particulate urease activity in their cell walls, induced by culturing algae on urea. About 88% of algal cells containing cell wall-urease bind purified ABP. This binding diminishes ABP arginase activity as well as urease activity in algal cell walls. No significant ultrastructural alterations of whole thallus or algal cells have been observed after incubation on urea.

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Zusammenfassung

MOLINA M. C., VICENTE C., PEDROSA M. M. & MUNIZ C. 1996. Die Bindung eines markierten Lectins der Flechte *Xanthoria parietina* an ihren eigenen Phycobionten und die Analyse seiner Enzymaktivität. – Phyton (Horn, Austria) 36 (1): 145–158, 4 Abbildungen. Englisch mit deutscher Zusammenfassung.

Ein teilweise gereinigtes Algen-bindendes Protein (ABP) wurde von Thalli der Flechte *Xanthoria parietina* gewonnen. Dieses Algen-bindende Protein verhält sich wie ein Phytohaemagglutinin gegenüber menschlichen Erythrocyten und besitzt eine Arginase-Aktivität. Rund 30% des ABP kann unter Verwendung gesättigter Konzentrationen von Ferritin oder Fluorescein durch zufällige chemische Interaktionen markiert werden. Markiertes ABP kann 24 bis 30% der *Xanthoria*-phycobionten binden, wenn sie eine bestimmte Urease-Aktivität in ihren Zellwänden aufweisen, welche durch Kultur der Algen auf Harnstoff induziert wurde. Rund 88% der Algenzellen, welche Urease in ihrer Zellwand enthalten, binden gereinigtes ABP. Diese Bindung verringert sowohl die Arginase-Aktivität der ABP als auch die Urease-Aktivität in den Zellwänden der Algen. In der Ultrastruktur des gesamten Thallus oder der Algenzellen konnten nach Inkubation durch Harnstoff keine signifikanten Veränderungen beobachtet werden.

Introduction

Lectins from lichens have been described for a long time (ESTOLA & VARTIA 1955, LOCKHART & al. 1978). However, BUBRICK & al. 1981 established for the first time the category of "recognition-type protein" for one of these proteins. BUBRICK & al. 1985 described a chemical procedure to obtain a partially purified algal-binding protein from *Xanthoria parietina* which contains at least two major proteins, one of those being able to bind to the lectins ConA from *Canavalia ensiformis*, and to RCA from *Ricinus communis*. Only isolated algal cells, axenically cultured for long time periods, are able to bind the algal-binding protein produced by their respective mycobiont (BUBRICK & GALUN 1980, GALUN 1990), whereas phycobionts recently isolated from their thalli do not bind the lectin. Recently, MOLINA & al. 1993 were able to find that ABP (algal-binding protein) from *X. parietina* binds to recently isolated phycobiont cells after a very short culture for 2 h on urea, although no more than 30% of the total phycobiont cells fluoresce. However, binding of ABP to its cell wall receptor completely inhibits urease activity in algal cell walls as well as arginase activity of this ABP. This is in agreement with the fact that many lectins have been identified as enzymes (SHANNON & HANKINS 1981) and it is related to the rationale which proposed that the role of lectins may not be in the species-specific recognition (LEROUGE & al. 1990) but in the mechanism of plant defense against potential parasites (CHRISPEELS & RAIKHEL 1991, FRANZ 1990), including lichens (AHMADJIAN 1987). Putrescine is clearly related to fungal infectivity (RAJAM & al.1985) and, in lichens, secreted fungal arginase could disorganize algal chloroplast and produce

chlorophyll degradation by increasing the level of putrescine in the phycobiont (VICENTE & LEGAZ 1983).

In this paper, we attempt to learn why only a third of XANTHORIA phycobionts is able to bind labelled ABP on the basis of protein composition of this partially purified protein.

Material and Methods

Abbreviations

ABP, algal-binding protein; ConA, concanavalin A; PBS, phosphate saline buffer; RCA, *Ricinus communis* agglutinin; SE-HPLC, size-exclusion, high performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

Plant material

Xanthoria parietina (L) Th. Fr., growing on *Robinia pseudoacacia* L. was collected in Montejo de la Sierra (Madrid). Thalli were air-dried and stored at 4 °C in the dark, no longer than two weeks.

Purification of *Xanthoria* ABP and its labelling

ABP was partially purified from *X. parietina* thalli according to BUBRICK & al. 1985, dispersed in PBS and labelled with fluorescein as described by MOLINA & al. 1993. ABP fraction was alternatively labelled with ferritin according to BAYER & al. 1976, modified for ABP by MOLINA & al. 1993. This labelled ABP was then filtered through a column of Sephadex G-150 (30 x 3.0 cm) equilibrated with PBS buffer. Protein eluted from 110 ml to 125 ml was used to label isolated algal cells.

The unlabelled ABP was filtered through a column (35 x 3.0 cm) packed with Sephadex G-150. The fraction which contained the highest amount of protein, defined as purified ABP, was dialyzed against distilled water and lyophilized to be analyzed by SE-HPLC.

Binding of labelled ABP to phycobiont cells

Three different samples were used to assay the binding of fluorescent, partially purified ABP (or ferritin-labelled ABP) to phycobiont cells. Thalli of *X. parietina*, recently collected (1.0 g of air-dried material), were incubated for 2 h on 20 ml of 40 mM urea in 75 mM potassium phosphate buffer, pH 6.9, in the dark, at 26 °C (RODRIGUEZ & VICENTE 1991). Thalli recently collected, without any treatment, were used as a control. Phycobiont cells were isolated from both samples according to ASCASO 1980. After mechanical disruption of thalli in distilled water, suspensions were centrifuged in a 0.5 M sucrose-80% (w/v) KI gradient. Algal cells that remained in the interface were collected, repeatedly washed with distilled water and used for lectin binding assays. A third sample was prepared by using isolated algal cells from untreated thalli which were then cultured for 2 h in 5.0 ml of 40 mM urea in 75 mM potassium phosphate buffer, pH 6.9, with continuous shaking. This last culture was repeated by shaking algal cells for 4 h in 5.0 ml of 100 mM urea when purified ABP was used. After culture, cells were repeatedly washed with distilled water and used for binding experiments. Fluorescent-labelled ABP was visualized by using a fluorescence light microscope.

Ultrastructural analysis to detect ferritin-labelled ABP was performed by softening labelled cells in a phenol:acetic acid (1:1, v/v) mixture for three days and then the material was fixed and dehydrated as described by ASCASO & GALVAN 1976. Embedding was done in Araldite resin for 3 days at 70 °C. Ultrathin sections (600 Å), obtained with an OmU2 Reichert Ultratome, were examined on a Philips E.M. 300 electron microscope operating at 80 kV.

Algal cell wall isolation and binding of ABP to isolated cell walls

When indicated, isolated phycobionts were ground in a mortar with 5.0 ml PBS buffer and the slurry centrifuged at 3200 g to isolate cell walls (BRUNNER & HONEGGER 1985). The pellet was washed with 5.0 ml acetone, disrupted in 2.5 ml PBS buffer at 20 KHz using a MSE sonic oscillator and centrifuged at 3200 g for 30 min at 2 °C (LEGAZ & VICENTE 1989).

Cell wall fractions obtained from phycobiont cells cultured for 2 h on 40 mM urea (or for 4 h on 100 mM urea) were resuspended in 3.0 ml PBS buffer to which 0.1 mg of unlabelled or fluorescein-labelled ABP was added and the mixture was incubated for 2 h at 26 °C. Finally, the mixtures were centrifuged at 3200 g for 15 min at 2 °C. Pellets were assayed for urease activity whereas supernatants were assayed for arginase activity.

Enzymatic activities of ABP and its receptor

ABP was assayed, before and after binding to algal cell walls, for arginase activity according to LEGAZ & VICENTE 1982. Protein was measured by the method of LOWRY & al. 1951, using bovine serum albumin as a standard. A unit of specific arginase activity is defined as 1.0 mmol of ammonia produced mg^{-1} protein min^{-1}.

Urease activity in the algal cell walls, before and after ABP binding, was mesured by the CONWAY 1962 microdiffusion method, according to LEGAZ & VICENTE 1989. A unit of specific urease activity is defined as 1.0 (mol of ammonia produced mg^{-1} protein min^{-1}. The amount of protein in the cell walls was measured according to LEGAZ & VICENTE 1989.

SE-HPLC separation of labelled ABP

Approximately 20 µg of total fluorescein-labelled protein was chromatographed on a 60 cm x 7.5 mm TSK-GEL HPLC column packed with G3000 PW, equilibrated with 10 mM Tris-HCl buffer, pH 9.15, using a Spectra Physics SP8800 liquid chromatograph equipped with a SP 4290 computer, according to PEDROSA & LEGAZ 1991. The same buffer was used as mobile phase. Detection was performed by using a Fluorichrom TM detector from Varian and a Spectra Physics SP8490 V-UV detector in series (MOLINA & al. 1993). When ferritin-labelled samples, previously filtered through Sephadex G-150, were analyzed, only the UV detector at 280 nm was used. Tyroglobulin (660 kDa), apoferritin (450 kDa), catalase (240 kDa), alcohol dehydrogenase (160 kDa), egg albumin (44 kDa) and cytochrome c (12.5 kDa), from Sigma Chemical Co., were used as molecular mass standards.

Electron microscopy

Whole thallus samples as well as algae isolated from untreated thalli were incubated in 40 mM urea for 2 h and later fixed in 3% (v/v) glutaraldehyde, dehydrated and embedded in Spurr's resin (SPURR 1969) and Araldite resin respectively, according to BROWN & al. 1987a. Ultrathin sections were obtained by

using an OmU2 Reichert ultratome, stained with lead citrate (REYNOLDS 1963) and observed under a Philips 350 electron microscope.

Phytohaemagglutinin assay

Two glass slides containing 30 µl of recently extracted human B- blood were placed under a light microscope and treated with 30 µl of saline solution (LOCKHART & al. 1978) or 30 µl ABP solution containing 0.37 mg protein ml^{-1}. Haemagglutination was observed for 15 min at 25 °C.

Results

Assay of haemagglutination

There was no visible haemagglutination in blood samples mixed with saline solution (Fig. 1A). However, haemagglutination was found from the first min in those samples mixed with partially purified ABP. Erythrocytes move towards the first agglutinated nodules. These increase in complexity and their size seemed a function of time, as it can be observed in Fig. 1B, corresponding to 5 min after ABP addition.

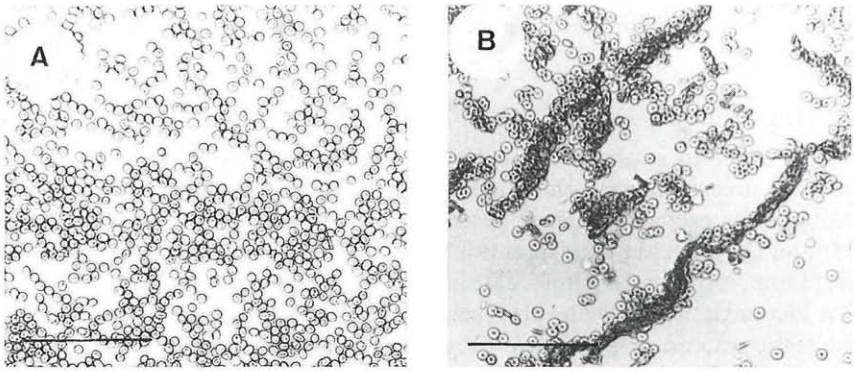

Fig. 1. Phytohaemagglutinin activity of ABP partially purified from *Xanthoria parietina* on B- human erythrocytes. A Control in saline solution. B Haemagglutination 5 min after adding ABP solution. Bars = 0.4 mm.

Labelling of phycobiont cells with ABP

When ferritin-labelled, partially purified ABP was added to algal cells isolated from thalli floated for 2 h on urea, a few cells, about 6% of the total, retained the label whereas phycobionts recently isolated from stored thalli did not contain significant label. In contrast, about 24% of visualized cells retain ferritin-labelled, partially purified ABP, bound to the cell wall surface when *Xanthoria* phycobionts were cultured for 2 h on urea (Table 1). Similar results were obtained by using fluorescein-labelled ABP.

Table 1

Quantitative analysis of dead and living algal cells and their labelling with ferritin after their culture on 40 mM urea for 2h. No significant differences for living and dead cells were observed for the different treatments. However, differences between treatments 1 and 3 for labelling were statistically significant (square chi proof)

Treatment	Algal cells number		
	living	dead	labelled with ferritin
1. Algal cells isolated from untreated thalli	49	15	4
2. Algal cells isolated from thalli floated on 40 mM urea for 2h	32	6	8
3. Algal cells isolated from untreated thalli and cultured for 2h on 40 mM urea	60	13	18

By considering fluorescent cells as living cells, according to MARX & PEVELING 1983, quantification of both dead and living cells was performed.

SE-HPLC analysis of labelled ABP

In order to explain why a low percentage of algal cells containing induced urease adsorbed labelled ABP, SE-HPLC analysis of the labelled protein was performed. Three peaks were revealed by monitoring eluates with an UV detector, with retention time values of 25.62 min, 27.1 min and 29.14 min, representing molecular masses ranging from 100 kDa to 25 kDa. The last peak, with a molecular mass of 25 kDa, represented about 30% of the total amount of protein (on the basis of the number of area counts) injected onto the column. However, only two peaks of protein with retention time values of 25.43 min and 27.87 min were revealed by monitoring eluates by fluorescence emission. Retention time values obtained by UV absorption were slightly higher than those obtained by fluorescence recording since the UV detector was placed behind the fluorescence detector. The purified, labelled ABP was also analyzed by SE-HPLC. In this case, only one peak with a retention time of 29.11 min was obtained by monitoring protein at 280 nm, or 27.91 min by fluorescence emission.

The ferritin-treated, partially purified ABP was firstly fractionated by passing the mixture through a Sephadex G-150 column in order to separate ferritin dimers from ferritin-ABP complexes. Material corresponding to a sharp peak, with coincident absorption maxima at 435 nm (ferritin chromophore) and 280 nm was eluted from 95 ml to 140 ml filtrate. This

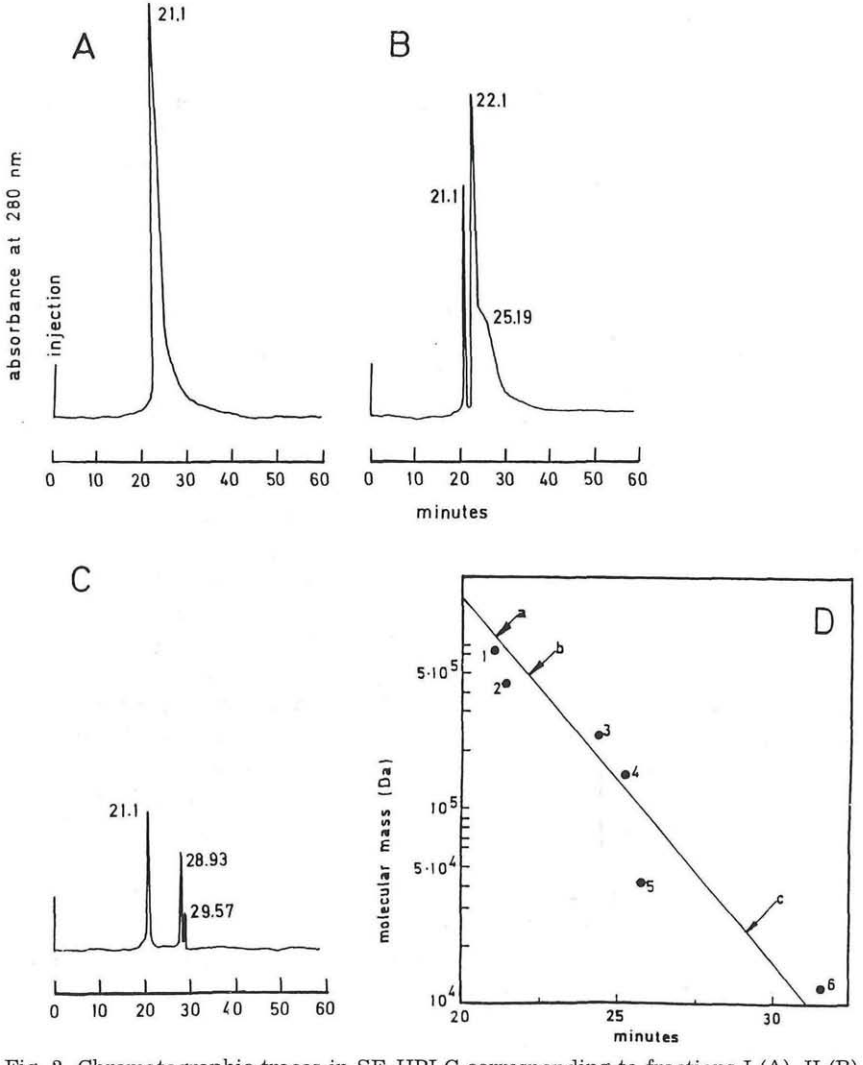

Fig. 2. Chromatographic traces in SE-HPLC corresponding to fractions I (A), II (B) and III (C) eluted from Sephadex G-150 column. Estimation of molecular weight (D) of ferritin-glutaraldehyde-ferritin (a), ferritin-glutaraldehyde-arginase (b) and free arginase (c) where standards were 1= tyroglobulin (660 kDa), 2 = apoferritin (450 kDa), 3 = catalase (240 kDa), 4 = alcohol dehydrogenase (160 kDa), 5 = egg albumin (44 kDa) and 6 = cytochrome c (12.5 kDa)

material was divided in four main sub-fractions. Fraction I consisted of protein eluted at 100 ml filtrate whereas fractions II, III and IV contained protein eluted from 105 ml to 110 ml, from 115 ml to 120 ml, and from

125 ml to 140 ml filtrate. Fraction I was resolved by SE-HPLC in only one peak with a retention time of 21.1 min (molecular mass 795 kDa), probably a dimer of ferritin (Fig. 2A). Fraction II was separated to give two main peaks (Fig. 2B) with retention time values of 21.1 min (dimer of ferritin) and 22.1 min, corresponding to a molecular mass of about 480 kDa (probably the ferritin-ABP complex), and a shoulder at 25.19 min. Ferritin dimers were the main component of fraction III, although a peak at 28.93 min (molecular mass of about 25 kDa) was also detected (Fig. 2C and 2D). However, this last peak represented only 1.3% of total ABP mixed with ferritin, calculated on the basis of peak area counts. Since no significant amount of protein was detected in fraction IV, about 98.7% of total ABP was recovered in fraction II although this did not imply that all ABP binds ferritin.

Enzymatic activity of ABP

Analysis of arginase activity related to the different labelling patterns is shown in Table 2. Arginase activity of fluorescein-labelled, partially purified ABP was reduced to 32.6% of that shown by the original, unmodified ABP. The loss of arginase activity found for fluorescein-labelled ABP can be clearly explained on the basis of the inhibitory action of fluorescein, tested by using a commercial, purified arginase. In contrast, the binding of ferritin to partially purified ABP only produced a loss of arginase activity of about 9.4% very similar to that shown after binding of ferritin to commercial pure arginase. However, arginase activity was completely lost when fluorescein-labelled ABP was adsorbed on isolated algal cell walls containing induced urease activity (Table 2). Urease activity contained in cell walls was completely lost after ABP binding.

However, urease in cell walls was only partially inactivated when they were incubated with purified ABP. This inactivation involved 34.1% of the total urease activity. In parallel, 23% of the original arginase activity of the purified ABP was lost after incubation of the lectin with its receptor (Table 3).

Ultrastructural alterations of algal cells during treatment

Whole thalli and algal cells isolated from untreated thalli were cultured for 2 h in 40 mM urea to study possible ultrastructural alterations induced by urea. Significant alterations in the ultrastructure of both algal and fungal cells (Fig. 3A and B) and even in the contact zone between alga and fungus (Fig. 3C) were not observed for whole thallus. Algal chloroplast proved to be unmodified as well as the arrangement of pyrenoglobuli to build the pyrenoid (Fig. 3D). In addition, no significant differences were observed in the isolated algae cultured for 2 h on urea (Fig. 4).

Table 2

Determination of arginase activity of free and labelled ABP fraction and urease activity of the ABP receptor. When unlabelled ABP was used, the initial amount of protein in contact with algal cell walls was 88 µg ABP. The value of 57 µg protein for the third treatment gives the amount of ABP remaining in the supernatant after cell walls separation by centrifugation of the mixture. For inhibition experiments, 2.0 mg fluorescein were included in the reaction mixture.

Preparation	ABP used in the reaction mixtures (µg)	Arginase activity (units)	Urease activity (milliunits)
Untreated, partially purified ABP	88	18.7	0.0
Untreated algal cell walls	–	–	41.0
Partially purified ABP adsorbed on algal cell walls	57	0.0	0.0
Fluorescein-labelled partially purified ABP	46	6.1	0.0
Ferritin-labelled ABP (Fraction II)	34	17.1	0.0
Ferritin-unlabelled ABP (Fraction III)	0.44	undetected	–
Commercial arginase	300	44.1	–
Fluorescein-treated commercial arginase	300	20.2	–
Ferritin-treated commercial arginase	300	40.1	–

Table 3

Determination of arginase activity of purified ABP and urease activity of ABP receptor. Induced cell walls always contained urease activity.

Matrix	ABP added (67 µg)	Urease activity (milliunits)	Arginase activity (units)
Induced cell walls	–	41.0	0.0
Non-induced cell walls	–	0.0	0.0
Induced cell walls	+	27.0	9.5
Non-induced cell walls	+	0.0	12.35

Discussion

ABP isolated and partially purified from *X. parietina* develops phytohaemagglutinin activity, a well known property of lectins (Lis & Sharon 1973). ABP is able to haemagglutinate human erythrocytes, as it

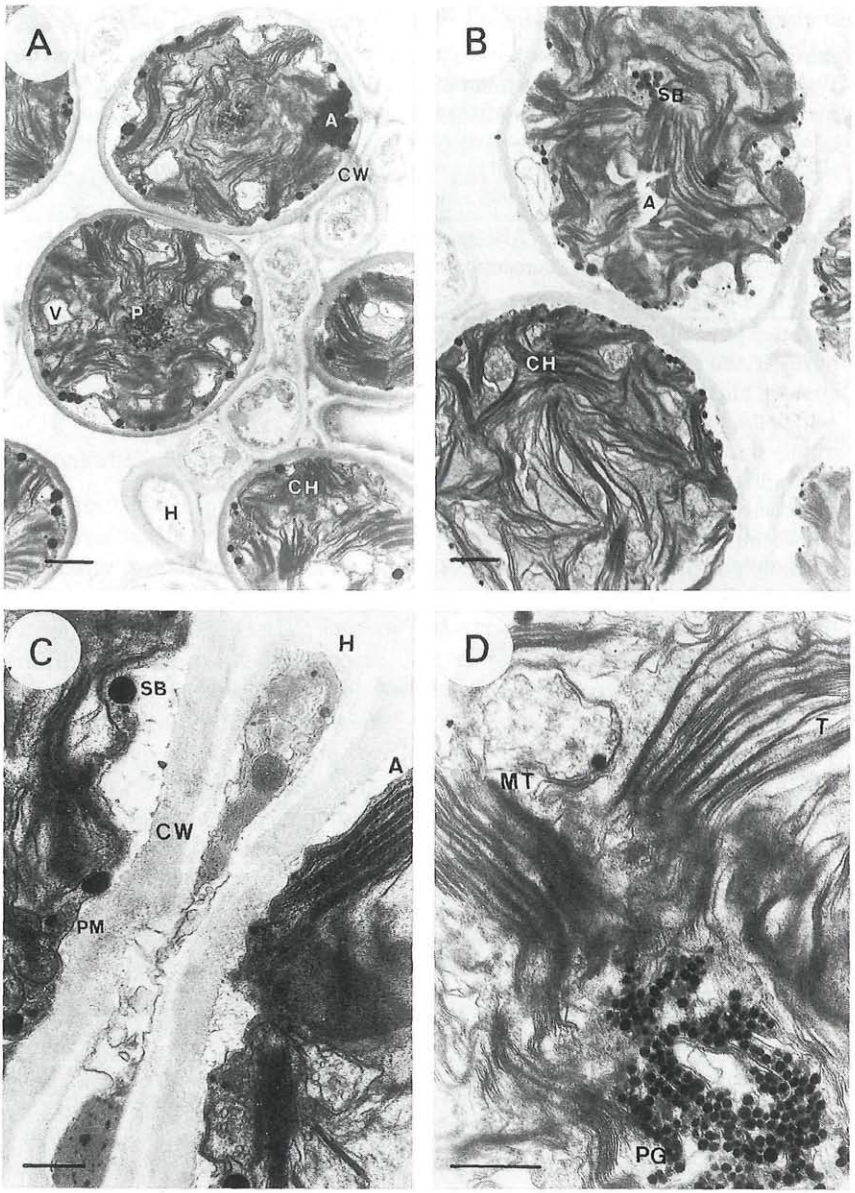

Fig. 3. Transmission electron microscopy of *Xanthoria parietina* thalli recently collected (A) or floated for 2h on 40 mM urea (B). Magnification of alga-fungus contact zone (C) and pyrenoid (D) are also shown. Bars = 2 µm (A and B) or 0.5 µm (C and D). CH = chloroplast; CW = cell wall; H = hypha; P = pyrenoid: PG = pyrenoglobuli; PM = plasmalemma; SB = storage bodies; T = thylakoid; A = algal cell.

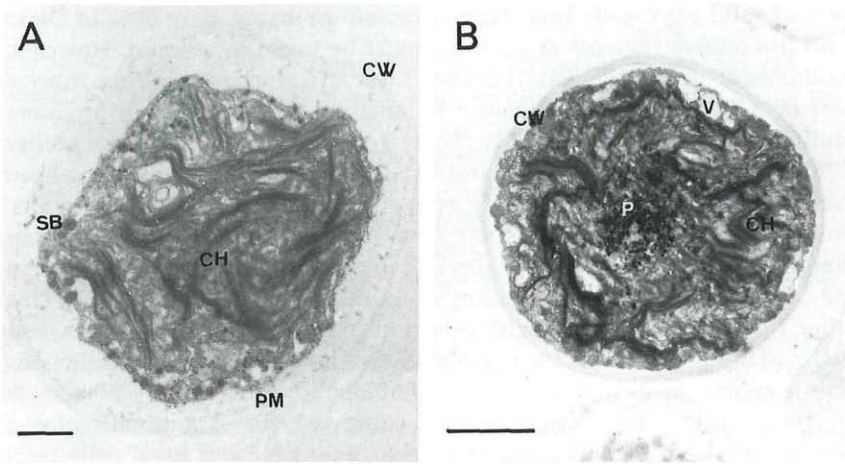

Fig. 4. Transmission electron microscopy of algal cells recently isolated from *Xanthoria parietina* thalli (A) or cultured for 2 h on 40 mM urea (B). Bars = 2.0 µm. Abbreviations as in Fig. 3.

has been shown in Fig. 1B. Haemagglutination nodules are not dissolved (SHANNON & HANKINS 1981) with time, but they increase in size by including new red cells. This fact could derive from the absence of glycosidase activity against erythrocyte membrane receptors. By contrast, partially purified ABP possesses another enzyme activity, such as arginase (Table 2), which is completely conserved after purification of ABP to homogeneity (Table 3). After adsorption of ABP on algal cell walls containing detectable urease activity, arginase activity of partially purified ABP was completely lost whereas that of purified ABP was partially lost (Tables 2 and 3). Since lectins bind to their cellular receptors by a glycosyl-glycosyl interaction (STOLL & al 1988), the loss of arginase activity must be due to a change of the tertiary structure of the protein, produced after binding. Differences in arginase activities between partially purified and fully purified ABP when they were adsorbed on cell walls containing urease could be interpreted as a dilution problem, since similar amounts of total protein were used (88 µg of partially purified and 67 µg of totally purified ABP) to bind to algal cell walls. According to this rationale, all urease activity might be lost upon pure arginase binding but about 65% of cell wall urease activity was recovered after arginase adsorption. From these results, it can be concluded that partially purified ABP contains other proteins, different from arginase, that bind to cell wall urease. Thus, arginase seems to be only one of several lectins contained in ABP.

The major problem derived from labelling experiments concerns the low percentage of algal cells which bind labelled ABP (Table 1). The possibility that a number of algal cells could bind unlabelled lectin if only

24% of total algal cells bind ferritin or, alternatively, only 29% of those bind fluorescein (MOLINA & al. 1993) must be taken in account. However, fundamental differences exist between these two kinds of labelling. Since a part of ABP consists of arginase, the labelling with fluorescein produces inhibition of the enzyme activity (Table 2). This does not affect the ability of fluorescein-labelled ABP to bind algal cell walls when urease has been induced, since fluorescence only appears on urease-positive cell walls. Since only 38% of arginase activity from ABP is retained after fluorescein binding, a low proportion of algal cells can retain unlabelled ABP, and then they are not visualized. In addition, both unlabelled and labelled proteins other than arginase (those with retention time values of 25.62 min and 27.1 min in Fig. 2A) could be retained by urease-containing algal cell walls. This is in agreement with the high level of labelling obtained by BUBRICK & al. 1982 for the same lichen species using polyclonal antibodies for a presumably heterogeneous ABP. However, about 88% of algal cells bind fluorescent, purified ABP. This fact can indicate that the occurrence of contaminating proteins in partially purified ABP could impede the binding of arginase (lectin) to its cell wall receptor.

Since total arginase activity of partially purified ABP was separated in only one fraction from Sephadex G-150, but it is apparently composed, at least, by two main fractions (retention time values of 22.1 min and 25.19 min in SE-HPLC), it must be concluded that the partial labelling of ABP with ferritin (Fig. 2) is a randomized consequence of the use of three different reactants, ferritin, glutaraldehyde and arginase (BAYER & al. 1976). Thus, complexes (ferritin-glutaraldehyde-ferritin, ferritin-glutaraldehyde-arginase, glutaraldehyde-ferritin, and glutaraldehyde-arginase) and free reactants can be produced (or recovered) with identical probability.

Incubation of thalli or isolated algae on urea could produce ultrastructural alterations in algal cells as a consequence of changes in the cellular osmotic pressure or those derived from the ammonia produced from urea hydrolysis inside the cell. However, neither algal structure nor physical contact between algal and fungal cells are modified by urea, as shown in Figs. 3 and 4. Changes in the arrangement of pyrenoglobuli (BROWN & al. 1987a) or collapsed cells (BROWN & al. 1987b) found for other lichen species after desiccation are not visualized after incubation of XANTHORIA thalli or its phycobiont on urea. In addition, urea does not produce significant changes in the content of chlorophylls of cultured phycobionts (MOLINA & VICENTE 1993).

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