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Macromolecules Associated with the Cell Walls of Symbiotic Dinoflagellates

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Abstract

One hypothesis often advanced to explain the observed specificity in algal-invertebrate symbioses is that macromolecules on the algal cell surface are ligands which interact with specific receptors on animal cell membranes. To initiate a test of this hypothesis, we have isolated cell walls from the coccoid stage of four symbiotic dinoflagellate species, and have found that they contain a calcofluor-positive component consistent with cellulose. In addition, SDS-PAGE analysis of solubilised fractions of the walls revealed several proteins/glycoproteins, ranging in molecular size from about 13.5 to > 200 kDa. This observation represents the first demonstration of proteins/glycoproteins associated with the cell walls of symbiotic dinoflagellates. We have also found that all four species of symbiotic dinoflagellate tested, release a range of water-soluble polypeptides/glycoproteins to the culture medium, raising the novel possibility that released macromolecules, rather than cell wall-associated components, could be the recognition "signal(s)" passing between symbionts and hosts.

Keywords: symbiosis, dinoflagellates, cell wall glycoproteins, extracellular glycoproteins, recognition

Abbreviations: SDS: sodium dodecyl sulphate; PAGE: polyacrylamide gel electrophoresis; PAS: periodic acid-Schiff reaction; HPLC: high performance liquid chromatography

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1. Introduction

In recent years, it has become increasingly apparent that symbiotic associations between marine invertebrates and dinoflagellates demonstrate a great deal of specificity (Trench, 1987). Although several different empirical studies have shown that different hosts show distinct preferences for a particular symbiont (Kinzie, 1974; Schoenberg and Trench, 1980b; Colley and Trench, 1983) and the same phenomenon is reflected in the natural distribution of symbionts in their receptive hosts (Trench, 1987), the mechanisms involved in the selection of an appropriate symbiont by its host are basically unknown (Trench, 1988).

Several hypotheses have been advanced to explain the observed specificity in symbioses between microalgae and invertebrates (see Trench, 1988 for review). Unfortunately, none of these hypotheses have been rigorously tested. One hypothesis that is repeatedly advanced pertains to macromolecules produced by the algae, associated with the cell wall, which interact with receptors associated with the animal plasmalemma (at the time of phagocytosis) or the vacuolar membrane (the symbiosome membrane) after phagocytosis. Unfortunately, as far as symbiotic dinoflagellates are concerned, there are no detailed studies of the chemical composition of the cell wall.

If ligand-receptor interactions do play a role in aspects of the "recognition" phenomenon in the establishment of dinoflagellate-invertebrate symbioses, then perhaps an appropriate point of departure in the analysis of such interactions should be an analysis of the cell walls of symbiotic dinoflagellates to determine if macromolecules, such as glycoproteins, usually associated with immunological and other recognition phenomena, are present. With this in mind, we have analysed the isolated cell walls from four symbiotic dinoflagellates, and find that in addition to cellulose, the walls do contain proteins and/or glycoproteins. In addition, and probably more significant, was the observation that all four species of algae tested released water-soluble, large molecular weight polypeptides/glycoproteins to the culture medium.

2. Materials and Methods

Maintenance of algal cultures

The algae used in this study are: *Symbiodinium microadriaticum* Freudenthal (*emend.* Trench and Blank, 1987), *S. kawagutii* Trench and Blank; *S. pilosum* Trench and Blank; and the *Symbiodinium* sp. (#175) isolated from the clam *Tridacna maxima* (Chang and Trench, 1982). These algae

have been maintained in axenic culture for several years. For analytical purposes, the algae were inoculated into sterile ASP-8A (see Blank, 1987) in 3L Fernbach flasks, to which ampicillin (50 $\mu g \cdot ml^{-1}$) was added, and grown at an irradiance of 80 $\mu E \cdot m^{-2} s^{-1}$ on a 14:10 hr (light:dark) photoperiod.

Harvesting cells and isolating cell walls

Algal cells were harvested by centrifugation at 6000×g. The supernatant was collected and saved. Algal pellets were resuspended in 10 ml TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and washed twice in TE buffer.

The pelleted cells in TE buffer were passed three times through a French Pressure cell at 110 M Pa and the resulting slurry centrifuged to 6500×g for 15 min at 0° C. The supernatant was discarded, and the pellet resuspended in 2 ml TE buffer, divided into 1 ml aliquots, and each mixed with 9 ml 80% (w/v) sucrose in TE, and centrifuged at 36000×g for 2 hr at 10° C. The recovered pellets were transferred to Eppendorf microfuge tubes in TE buffer and washed several times by centrifugation and resuspension in TE buffer.

Cellulase digestion of cell wells

Isolated cell walls were extracted with methanol (1 hr), methanol:chloroform (2:1, 1 hr) and methanol (1 hr) and washed twice with distilled water (Loos and Meindl, 1982) before treatment with 69 U/ml cellulase CEL (Worthington Biochemical Corp.) in 100 mM acetate buffer (pH 4.7) at 37° C for 24 hr. Untreated controls and treated cell wall preparations were stained with Calcofluor white M2R and observed with an epifluorescence microscope.

Extraction of cell walls

Isolated cell walls were extracted with 1% (w/v) SDS and 2% (v/v) β-mercaptoethanol at 37° C for 18 hr. After centrifugation at 15000×g, the supernatant was recovered as SDS-soluble material, and the pellet was washed three times with distilled water, and then extracted with 1 N NaOH at 25° C for 18 hr. After centrifugation at 15000×g the hydroxide-soluble supernatant was exhaustively dialysed against 1% (w/v) SDS. Samples of extracted proteins were separated by SDS-PAGE using gradient gels (5–20% acrylamide) and a Laemmli (1970) discontinuous buffer system. Gels were fixed and stained with silver (Merril et al., 1984).

Processing the extracellular fraction

The culture media recovered after harvesting cells were exhaustively dialysed against distilled water and lyophilized. This material was stored frozen at −20° C until analysed by SDS-PAGE as described above.

Incubations with $NaH[^{14}C]O_3$

Cells in log-phase growth (conditions as described above) were provided with $NaH[^{14}C]O_3$ at an initial concentration of 1.0 μ Ci·ml^{-1}. After 3 weeks of growth, the cells were harvested, and media and cell wall fractions processed as described above. After electrophoresis, the gells were stained with silver, dried, and the radioactivity present detected by autoradiography.

Electron microscopy

S. microadriaticum cells were fixed in 2% (v/v) glutaraldehyde in sea water for 4 hr, post fixed in 2% (w/v) OsO_4 in sea water, washed, dehydrated and embedded in Spurr's medium as previously described (Colley and Trench, 1985). Isolated cell walls, before and after cellulase digestion, were fixed and embedded by standard procedures. Ultrathin sections were viewed and photographed with a Seimens Elmisop I or a Phillips 200.

3. Results

Ultrastructure of cell walls

The ultrastructure of the cell wall of *S. microadriaticum* (Fig. 1a) indicates a thin outermost layer, and a thick, fibrous layer beneath (cf. Trench et al., 1981; Trench and Blank, 1987). Examination of the isolated cell wall by electron microscopy revealed essentially the same structures (Fig. 1b), but the thin outermost layer was absent. After digestion of isolated walls with cellulase, the prepartions indicated the loss of the fibrous component of the wall, and a resultant loss of structural integrity. Most living intact *Symbiodinium* cells do not stain with Calcofluor White, and cellulase treatment of living cells, or glutaraldehyde-fixed cells, does not result in digestion of the cell wall (Trench and Blank, 1987).

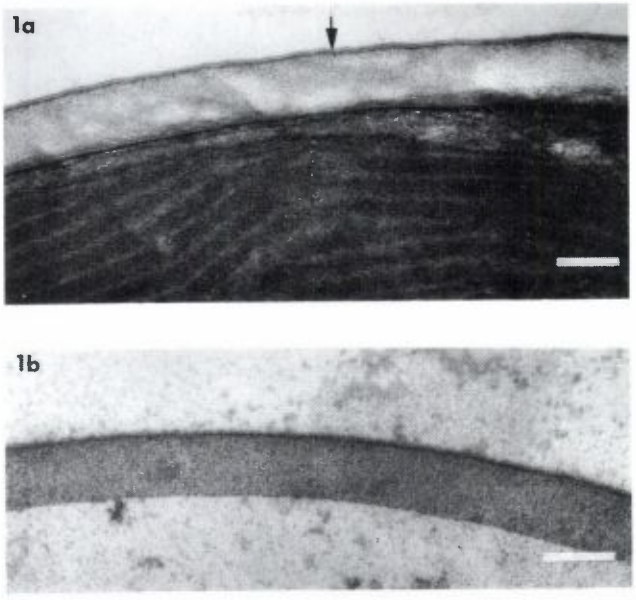

Figure 1. (a) Electron micrograph of the cell wall of *S. microadriaticum* in culture. Arrow indicates the outermost layer of the cell wall, the location of the putative sporopollenin. Scale bar = 0.02 μm.
(b) Electron micrograph of the isolated cell wall from *S. microadriaticum*. Note that the outermost layer (indicated by arrow in Fig. 1a) is missing in this preparation. Scale bar = 0.02 μm.

Calcofluor staining of the isolated cell walls

Preparations of isolated cell walls were stained with the fluorescent dye Calcofluor White and examined with an epifluorescence microscope. Calcofluor staining of control untreated cell wall preparations produced very strong fluorescence as illustrated in Fig. 2. Treatment of isolated cell walls with cellulase, before calcofluor staining, eliminated the fluorescence relative to control cell wall preparations not treated with cellulase. Calcofluor is believed to be specific for β-1,3 and β-1,4 glycosidic linkages (Hughes and McCully, 1975); the absence of calcofluor binding in the cellulase-treated samples is consistent with the cell walls being composed in part of a cellulose-like material. This interpretation is further strengthened by the observation that after cellulase digestion

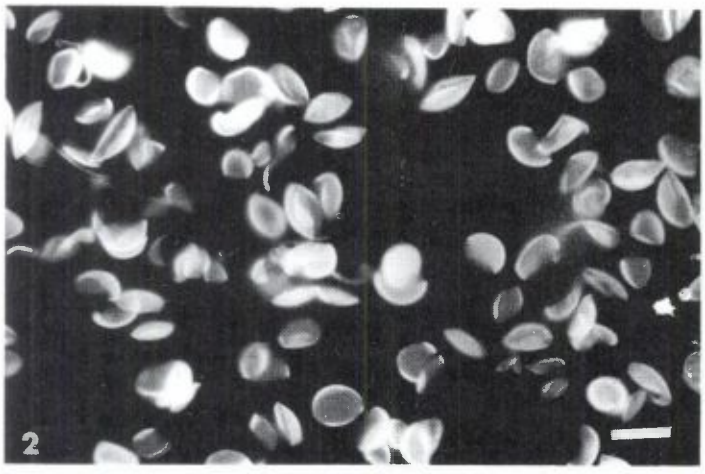

Figure 2. Epifluorescence micrograph of the isolated cell wall preparation from *S. microadriaticum* stained with Calcofluor white. Scale bar = 0.02 μm.

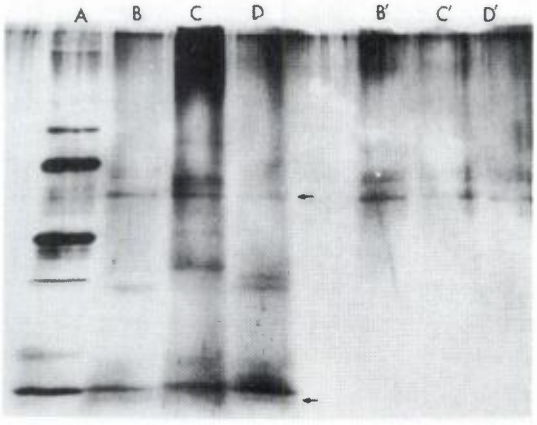

Figure 3. SDS-PAGE separation of solubilised cell wall polypeptides and glycopeptides from three symbiotic dinoflagellates. Lane A, molecular weight standards [(from top to bottom) phosphorylase b, 97,400; bovine serum albumen, 66,200; ovalbumin, 42,699; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400]. Lanes B,C and D, SDS-soluble fractions from *S. microadriaticum*, *S. kawagutii* and *S. pilosum*, respectively. Lanes B′, C′ and D′, NaOH-soluble fractions from the same algae.

of cell walls, the major released sugar detected by high performance liquid chromatography (HPLC) was glucose.

Electrophoretic analysis of cell wall proteins

To test the hypothesis that cell walls of symbiotic dinoflagellates contained proteins or glycoproteins, purified cell walls were first extracted with 1% SDS, the insoluble remains pelleted by centrifugation, and the pellet extracted in 1 M NaOH. The SDS-soluble and hydroxide-soluble fractions were analysed by SDS-PAGE.

The SDS-soluble fractions of cell walls from *Symbiodinium microadriaticum*, *S. kawagutii* and *S. pilosum* yielded from 9 to 14 discrete polypeptides, ranging from 14 to 200 kDa (Fig. 3). In the case of *Symbiodinium* sp. from *T. maxima*, about 8 polypeptide species were observed (Fig. 4, lane C). In all cases, large molecular species remained at the stacking gel-resolving gel interface. Although there were polypeptides with similar molecular sizes in the walls of all four algal species, there were some that were unique to each. The hydroxide-soluble fractions yielded 3 major (and frequently 2 minor) polypeptides, with

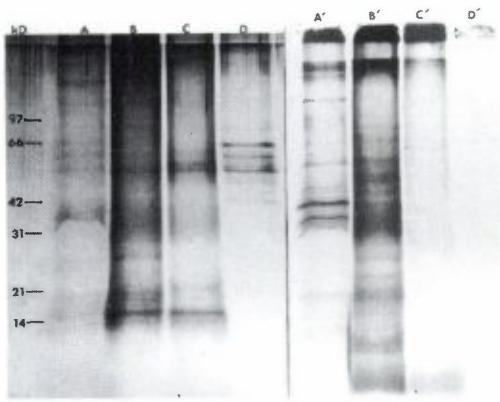

Figure 4. SDS-PAGE separation of components of the extracellular glycoproteins released by *Symbiodinium* sp. (from *Tridacna maxima*). Lane A, extracellular glycoproteins; Lane A′, autoradiogram of lane A, Lane B, SDS wash of cells; Lane B′, autoradiogram of lane B; Lane C, SDS extract of cell walls, Lane C′, autoradiogram of lane C. Lane D, NaOH extract of cell walls; Lane D′, autoradiogram of lane D. Apparent molecular sizes are given at the extreme left are based on the same standards given in Fig. 3 above.

very similar molecular sizes in all cases, in the 42 to 66 kDa range (Fig. 3 and Fig. 4D).

Several of the resolved polypeptides, and polypeptides that did not penetrate the resolving gel (but did penetrate the stacking gel) stained positively with PAS, an indication that the polypeptides are glycosylated. The only detected contaminant of the cell wall preparations was the pyrenoid. As a result, polypeptides with apparent molecular weights of 55 kDa and 12 kDa (arrows, Fig. 3) respectively, consistent with the large and small subunits of ribulose bisphosphate carboxylase-oxygenase were resolved. However, we have not conducted any further analysis, e.g. immunoblots, to determine the identity of these polypeptides. No other contaminant was detected.

Detection of released polypeptides by Symbiodinium *sp. from* Tridacna maxima

The possibility that the molecules responsible for "recognition" of symbiotic algae by hosts are released by the algae, rather than being an integral component of the cell wall, has not previously been tested. To test the hypothesis that symbiotic algae release proteins or glycoproteins, we grew *Symbiodinium* sp. (from *Tridacna maxima*) axenically in the presence of $NaH[^{14}C]O_3$ for 3 weeks, and harvested the cells by centrifugation at 20,000×g. The culture medium was saved as a lyophilized sample, dialysed against distilled water and then rendered 10 mM with respect to Tris-HCl (pH 7.1) and 0.1% SDS. The intact algae were washed with 0.1% SDS in sea water, and the wash dialysed against Tris-HCl (as above). The cell walls were isolated as described above, and SDS-soluble and hydroxide-soluble fractions prepared. Polypeptide components in the various fractions were separated by SDS-PAGE, and the radioactivity detected by autoradiography of the dried electrophoretograms.

The stained gel showed that the medium in which the cells were grown contained several polypeptides ranging in size from less than 14 to larger than 200 kDa (Fig. 4, lane A) and the autoradiogram of this sample (Fig. 4, lane A′) resolved that all silver-stained polypeptides were radioactive. Because of the increased sensitivity, the autoradiogram resolved several polypeptide species that were not clearly seen on the silver-stained gels. The autoradiogram also demonstrated that there were very large molecules that did not enter the resolving gel.

Both the silver-stained gel (Fig. 4, lane B) and the autoradiogram (Fig. 4, lane B′) of the SDS-wash of intact cells resolved several polypeptides, and again the autoradiogram indicated the presence of several more polypeptides than seen on the silver-stained gel. Again, there were large molecular weight

species that did not enter the resolving gel. In addition, several polypeptides, for example the major species at about 15 kDa, did not incorporate ^{14}C.

The SDS-extracted cell wall fraction from the algae from *T. maxima* (Fig. 4, lane C) yielded polypeptides that were similar to those seen in similar cell wall extracts from *S. kawagutii* (Fig. 3, lane C). Some of the polypeptides resolved were of similar molecular size to those resolved in the SDS wash of the intact cells. The autoradiogram of the SDS cell wall extract indicated that few of the polypeptides resolved by silver staining incorporated ^{14}C (Fig. 4, lane C′).

The silver-stained gel of the hydroxide-soluble fraction resolved polypeptides of similar molecular size to those observed in similar fractions of the cell walls from *S. microadriaticum*, *S. kawagutii* and *S. pilosum* (Fig. 4, lane D). Except for the component that did not enter the resolving gel, the autoradiogram indicated that none of the separated polypeptides incorporated ^{14}C (Fig. 4, lane D′). The reason for this observation is either that (a) under the conditions of the "pulse-chase" labelling procedure of the experiment, this pool did not incorporate ^{14}C, or (b) that these polypeptides represent a mobile pool, and the ^{14}C moved through it rapidly. We can not currently distinguish between these alternatives.

4. Discussion

The results of our study indicate that the cell walls of the coccoid stage of symbiotic dinoflagellates in the genus *Symbiodinium* are composed of cellulose and proteins. The observations that (1) cellulase digestion of cell walls yielded glucose as the major sugar after HPLC analysis, (2) calcofluor binds strongly to isolated cell walls, (3) the staining disappears, and the ultrastructural integrity of the wall is lost after cellulase treatment, are consistent with the interpretation that cellulose represents a major structural component of the cell wall. Yonge (1931), using histochemical methods (iodine-sulfuric acid, chlorzinc iodide), had concluded that the "zooxanthellae" freshly isolated from *Galaxea fascicularis* possessed a cellulosic wall. Subsequent electron microscopic studies of "zooxanthellae" *in hospite* suggested that the wall was either very reduced, presumably to facilitate metabolite transport (e.g. Taylor, D.L., 1968; Kevin et al., 1969) or was clearly discernible (Dodge, 1973). Ultrastructural studies of the algae in culture (Schoenberg and Trench, 1980a; Trench and Blank, 1987) clearly demonstrated the presence of a robust cell wall enveloping the coccoid algal cells. It is of interest to note that the continuous walls of the coccoid stages ("vegetative cysts", Taylor, F.J.R., 1978) in the life history of these symbiotic dinoflagellates is comprised of a robust structure of cellulose and protein, while the amphiesma of the motile gymnodinioid "mastigote"

stages is comprised of a series of vesicles with rather insubstantial plates of unknown chemical composition (Trench and Blank, 1987). In the case of the armored mastigote dinoflagellates, the amphiesmal plates are believed to be cellulosic (Loeblich, 1969; Taylor, F.J.R., 1978).

The observations that calcofluor staining and cellulase digestion are both ineffectual in living undamaged cells and in glutaraldehyde-fixed cells, indicate that some component of the cell wall resists the penetration of the dye and the enzyme. The most likely candidate is the thin outermost layer observed (Fig. 1). Although we do not know the composition of this material, it is possible that it may be sporopollenin (Loeblich, 1984).

As far as we are aware, this paper represents the first demonstration of the presence of proteins/glycoproteins associated with the cell walls of symbiotic dinoflagellates. However, there are several reports of proteins/glycoproteins associated with the cell walls of other microalgae, which may or may not possess cellulosic walls (Lamport and Miller, 1971; Northcote et al., 1958; Thompson and Preston, 1967). In *Chlamydomonas reinhardii*, Catt et al. (1976) found the cell wall to be 70% glycoprotein, containing high levels of hydroxyproline, arabinose and galactose. Similarly, Miller et al. (1974) has reported a hydroxyproline-rich glycoprotein wall in *C. gymnogama*, where the hydroxyproline is glycosidically linked to heterosaccharides composed of arabinose and galactose. Analyses of the sugar and amino acid composition of the cell walls of several symbiotic dinoflagellates by HPLC are currently underway in our laboratory.

Although the cellulosic cell walls of symbiotic dinoflagellates have now been shown to be associated with proteins/glycoproteins, whether these proteins play a role in the "recognition" of symbiont by host remains unresolved. Three algal species tested, *S. microadriaticum*, *S. kawagutii* and *Symbiodinium* sp. from *T. maxima*, infect *Cassiopeia xamachana*, but *S. kawagutii* does to persist; *S. pilosum* does not infect. If the cell wall proteins/glycoproteins were involved in "recognition", it would be reasonable to expect a greater similarity between the three algal species that do infect the host. We could not discern such similarities. In fact, there are more polypeptides (with regards to molecular size) common to *S. microadriaticum* and *S. pilosum* than to *S. microadriaticum* and *S. kawagutii*. The polypeptide profiles of the hydroxide-soluble fractions were the same for all four species of algae.

The novel observation that symbiotic dinoflagellates grown in culture release large molecular weight glycoproteins raises the intriguing possibility that these molecules, rather than those that are integral components of the cell wall, represent the "recognition" signal(s) passing between symbiont and host, assuming that the same phenomenon occurs *in hospite*. That macromolecules

are released by the algae, and are not a result of lysis in culture, is supported by two observations; (a) that the water-soluble peridinin-chlorophyll *a*-proteins (PCP), an abundant an obvious intracellular constituent of the algae (Chang and Trench, 1982), was never detected in the medium, and (b) that anti-PCP antibodies (Govind et al., 1990) did not cross-react with any component of the exudate in immunoblot assays.

Glycoproteins, including lectins, are known to mediate various developmental processes in widely divergent groups such as algae (Gilles et al., 1984) and vertebrate cells (Erickson, 1989; Hemler, 1990), and have been hypothesized as playing a role in the establishment of species-specific symbioses (Smith and Douglas, 1987; Trench, 1988). It is obviously necessary to demonstrate that these glycoproteins observed in culture are released by the algae inside their hosts' cells. To this end, we have prepared polyclonal antibodies against the released glycoproteins from one species of symbiotic dinoflagellate, and through the technique of immunogold localization, we anticipate resolving whether the glycoproteins are detectable at the host-symbiont interface.

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