Polysaccharide localization in the sporophyte cell wall of *Adenocystis utricularis* (Ectocarpales *s.l.*, Phaeophyceae)

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The chemical composition of the sporophyte cell wall of *Adenocystis utricularis* was examined by using a number of histochemical techniques for the localization of polysaccharides, including labeling with a fluorescent alginate probe. Histochemical staining allowed confirmation of a low alginate/fucoidan ratio in the cell wall indicated by exhaustive extraction of polysaccharides from the algal thalli. Alginate was located in the inner portion of cell walls. ''Egg-box'' structure of alginate in inner cortical cells may be related to the mechanism of algal growth. Abundance of fucoidan was confirmed by extraction on semithin sections and staining. Weak staining on nonextracted material was in agreement with previously proposed blockage of sulfate groups in these polysaccharides.

KEY WORDS: *Adenocystis utricularis*, Alginate, Fucoidan, Histochemistry, Cell wall polysaccharides

INTRODUCTION

Adenocystis utricularis (Bory de Saint-Vincent) Skottsberg is a member of Phaeophyceae, which grows on rocks and in tidal pools of the littoral zone of the sub-Antarctic circumpolar region. The sporophytic macrothalli are hollow and bladderlike, bearing numerous cryptostomata containing hyaline hairs. A trichothallic apical meristem is responsible for the multiaxial thallus structure, made of a solid cortex and a fluid-filled medulla penetrated by a network of longitudinal filaments (Rousseau *et al.* 2000). Unilocullar sporangia are formed on the saccate macrothalli in spring and summer, whereas the species persists as discoid–filamentous gametophytic microthalli during the cold season (Müller 1984; Rousseau *et al*. 2000). Culture studies on its life history were performed by Müller (1984). To make clear its taxonomic position, Clayton (1985) carried out a critical investigation of the vegetative anatomy and growth of *A. utricularis*. Recently, Asensi *et al*. (2003, 2004), comparing natural populations, laboratory cultures and herbarium material together with data from partial ribosomal DNA large subunit sequencing, concluded that the genus *Adenocystis* should include three species: *A. utricularis* (Bory de Saint-Vincent) Skottsberg (type species), *Adenocystis rimosa* (Montagne) Asensi, Delépine, Reviers & Rousseau and *Adenocystis longissima* (Skottsberg) Asensi, Delépine, Rousseau & Reviers.

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Brown algae have been reported to produce alginates, laminarans and fucoidans as the main polysaccharides (Percival 1979; Painter 1983; Mabeau & Kloareg 1987; Chevolot *et al*. 2001; Duarte *et al*. 2001; Obluchinskaya *et al*. 2002; Bilan *et al*. 2006). The fucoidans of *A. utricularis* were recently characterized in our laboratory (Ponce *et al.* 2003). They presented important antiherpetic activity. The knowledge of the subcellular localization of the different polysaccharides in the algal thallus is an important way to check the efficiency of the different extraction procedures attempted in the production of substances of biological or pharmacological interest. Previous work on localization of polysaccharides in the cell walls of Phaeophyceae has been carried out by means of staining for optical microscopy (McCully 1970; Evans & Holligan 1972) and fluorescent labeling and immunocytochemistry (Vreeland & Laetsch 1989; Chi *et al*. 1999), but to the best of our knowledge none of these previous studies have dealt with *A. utricularis*. The aim of the present work is to describe the fine structure of *A. utricularis* sporophyte cell wall and to localize, by means of different histochemical techniques, cell wall polysaccharides in semithin thallus sections before and after extraction of fucoidans and alginic acids.

MATERIAL AND METHODS

Adenocystis utricularis thalli were collected in spring (October 2002) at La Lobería (46°08′S, 67°40′W), Santa

Cruz, Argentina. They grew attached to rocky tidal pools through a small disc-shaped basal holdfast, the globose thalli reaching up to 3 cm. Sections *c.* 1 mm^2 were fixed overnight in 2% glutaraldehyde with 0.1% caffeine in filtered seawater at room temperature. Postfixation proceeded in 1% OsO_4–0.005% potassium ferricyanide for 2.5 h, followed by dehydration in acetone series and inclusion in Spurr's resin. Sections were obtained with a Reichert microtome. Semithin sections (0.5–2 μm) were collected with a wire loop and fixed to microscope slides on a hot plate at 60°C. Ultrathin sections were collected on Formvar coated grids and stained with (1) uranyl acetate and lead citrate; (2) ruthenium red (carboxylated polysaccharides, Krishnamurthy 1999) and (3) PATAg (silver proteinate method, modification of periodic acid–Schiff (PAS) polysaccharides with free vicinal OH groups, Roland & Vian 1991). In the case of PATAg, oxidation with periodic acid was carried out for at least 3 h. Sections were observed in a JEOL 100CX-II transmission electron microscope (TEM) (Jeol, Akishima, Tokyo, Japan) at the Centro Regional de Investigaciones Básicas y Aplicadas de Bahía Blanca. The nature of cell wall polysaccharides was histochemically determined using various specific reactions: (1) metachromatic staining with 0.05% toluidine blue O (TBO) at pH 4.4 (sulfated and carboxylated polysaccharides) and pH 1.5 (sulfated polysaccharides, McCully 1970); (2) fluorescent brightener Calcofluor White [cell wall (1–4)- and (1–3)-β-D-glucans, Krishnamurthy 1999]; (3) berberine (heparin and polyphenols, Lulai & Morgan 1992; Stockert 2000) and (4) PAS after aldehyde block with 2,4-dinitrophenylhydrazine (Jensen 1962).

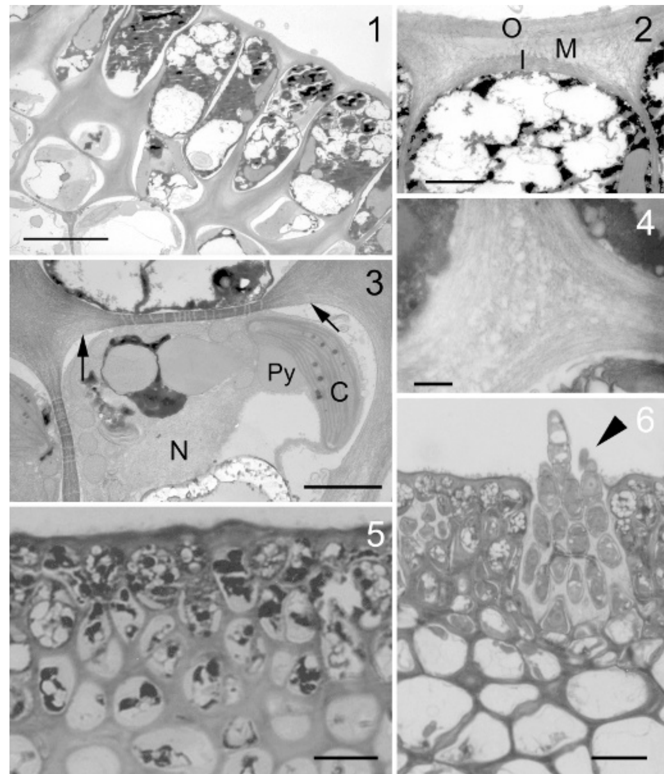
Extractions of fucoidans and alginic acids were performed on semithin sections with 4% H_2SO_4 at 70°C for 14 h and 1 M Na_2CO_3 at 70°C for 24 h, respectively (McCully 1970).

Fluorescent alginate probe synthesis (Vreeland & Laetsch 1989): Carboxyl moiety in alginic acid (Sigma) was coupled to 5-aminofluorescein in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide at pH 4.7. The conjugate was recovered by precipitation and centrifugation, dried in acetone and kept at −20°C until used. Alginate fluorescent probe gave a single absorption peak at 492 nm.

Probe incubation: Fluorescent probe (0.25 and 0.5 μg ml^{-1}) was dissolved in 50 mM Tris buffer pH 7.6 and incubated 2 to 12 h in darkness with (1) EDTA (0.25, 0.5 and 1 mM) (blank); (2) 0.25 mM EDTA and Ca^{2+} (1, 4 and 40 mM) and (3) 0.25 mM EDTA and Na^+ (150 mM). Sections were washed with 0.25 mM EDTA before observation.

RESULTS

Ultrathin sections of mature thalli of *A. utricularis* sporophytes showed that the epidermal cell wall consisted of an outer layer with fine, parallel fibrils, an amorphous mid-layer with disordered fibrillar material and an inner layer with fibrillar material in parallel arrangement (Figs 1, 2). Plasmodesmata were usually gathered in areas where the cell wall was thinner (Fig. 3). The cell wall adjacent to pore



Figs 1–6. Sporophyte cell wall of *Adenocystis utricularis*.
Figs 1–4. Transmission electron micrographs.
Fig. 1. General view of cortical cells in transverse section. Scale bar = 10 μm.
Fig. 2. Detail of epidermal cell wall to show the outer layer, the mid-layer and the inner layer. Scale bar = 2 μm.
Fig. 3. Detail of a subcortical cell. Longitudinal view of plasmodesmata between cells. The arrows indicate the thick cell wall at the corners of the cells. Scale bar = 2 μm.
Fig. 4. Detail of a portion of the wall among cortical cells stained with ruthenium red.
Figs 5–6. Light micrographs of transverse sections of thalli. Cortical cells stained with toluidine blue at pH 1.5 and 4.4, respectively. Note in Figure 6 a cryptostomata with hyaline hairs (arrowhead). Scale bar = 10 μm. C, chloroplast; I, inner layer; M, mid-layer; N, nucleous; O, outer layer; Py, pyrenoid.

areas appeared thicker because of a less compact packing of the fibrillar material (Fig. 3, arrows). A similar arrangement of fibrillar and amorphous polysaccharides was observed when contrasting with ruthenium red (Fig. 4). Notably, cell wall contrast was not intense.

Toluidine blue staining in nonextracted material at pH 1.5 and 4.4 rendered acid metachromasia (purple coloration) of the cell walls that was especially intense in the outer layer of the epidermal cells (Figs 5, 6). At pH 1.5, after fucoidan acid extraction in sections (Table 1), only a weak purple-violet staining was obtained, whereas alkali-extracted sections showed abundant purple-coloured material in cell walls. On the other hand, alkali-extracted sections stained at pH 4.4 preserved intense coloration. Sequential acid–alkaline extractions removed staining at both pHs.

Berberine gave strong fluorescence. Thus, fibrillar and matricial cell wall polysaccharides could not be clearly distinguished. The periclinal wall of the epidermal cells showed a stronger fluorescence, whereas it was weaker in

Table 1. Toluidine blue O staining at pHs 1.5 and 4.4 on nonextracted and alkali- or acid-extracted thallus sections of *Adenocystis utricularis*.

Treatment	pH	Observations
Nonextracted	1.5	Purple metachromasia in cell walls and mucilage; basophylic reaction in intracellular contents
Nonextracted	4.4	Purple metachromasia in cell walls and mucilage; basophylic reaction in intracellular contents
Residue after acid extraction	1.5	Very weak purple metachromasia in cell walls
Residue after alkaline extraction	1.5	Intense purple metachromasia in cell walls
Residue after sequential acid–alkaline extraction	1.5	No staining
Residue after alkaline extraction	4.4	Intense purple metachromasia in cell walls
Residue after sequential acid–alkaline extraction	4.4	No staining

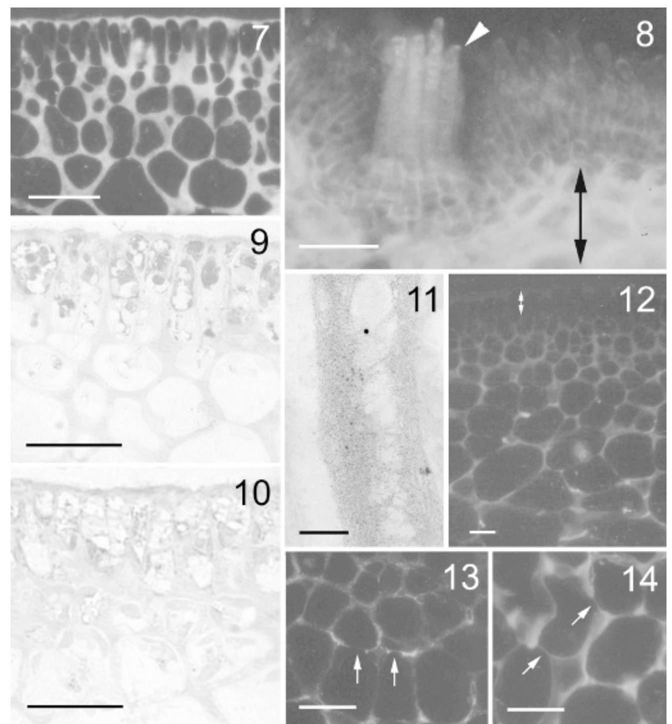
the anticlinal walls; fluorescence was also strong in the cortical cell walls (Fig. 7). Cell walls developed very weak autofluorescence under ultraviolet light but were fluorescent after applying the brightener Calcofluor White. Fluorescence was stronger in walls of inner cortical cells (Fig. 8, double arrow).

No significant difference could be detected between controls and periodic acid-oxidized sections with PAS staining for optical microscopy (Figs 9, 10). When observed with TEM, only a faint PATAg reaction revealed the fibrillar material and the amorphous material showed no silver deposits (Fig. 11). In controls (without periodic acid oxidation) the walls did not show silver deposits (not illustrated).

Labeling of semithin thallus sections with 5-aminofluorescein (AMF)-alginate probe was restricted to cell wall material; no fluorescent mark was observed intracellularly (Figs 12–14). Epidermal cell walls practically did not bind the fluorescent probe (Fig. 12, double arrow). Fluorescence was only obtained in presence of Ca^{2+}; no binding was evidenced in the presence of Na^{+}, whereas repeated washing with EDTA eliminated the fluorescent mark. When Ca^{2+} concentration increased from 4 to 40 mM the fluorescent mark increased, but always within the cell walls (Figs 13, 14 respectively). As expected, when increasing Ca^{2+} concentration some unspecific precipitation of the fluorescent label was observed because of the formation of insoluble calcium alginate. Cross-walls at plasmodesmata areas always denoted less intense labeling (Figs 13, 14, arrows), especially with 40 mM Ca^{2+}.

DISCUSSION

At pH 4.4, ionization of carboxylic and sulfate groups is responsible for purple metachromasia with TBO, whereas at pH 1.5 acid metachromasia is only due to the ionization of sulfate (McCully 1970; Krishnamurthy 1999). The latter groups are constituents only of fucoidans, whereas carboxylic groups are present both in fucoidans (as glucuronic acid) and alginic acid (as guluronic and mannuronic acids). Metachromatic staining with TBO in *A. utricularis* thalli showed no predominance of sulfated or carboxylated polysaccharides in any region, as was reported by Evans & Holligan (1972) in *Dictyota*. In *A. utricularis*, cell wall fucoidans would mainly contribute to the metachromasia at pH 1.5. Though it should be noted that the intensity of staining is weaker at this pH, low fucoidan content is not the cause since Ponce *et al.* (2003) obtained abundant fucoidan from these algal thalli, even after several aqueous extractions. Moreover, the results obtained with TBO at pH 1.5 in acid- and alkali-extracted thallus sections



Figs 7–14. Sporophyte cell wall of *Adenocystis utricularis*. Histochemical staining.

Figs 7–10. Micrographs of transverse sections of thalli.

Fig. 7. Fluorochrome berberine. Note weak fluorescence in the anticlinal walls of the outer cortical cells. Scale bar = 10 µm.

Fig. 8. Fluorescent brightener Calcofluor White. Note stronger fluorescence in walls of inner cortical cells (double arrow). The arrowhead shows a cryptostoma. Scale bar = 10 µm.

Figs 9–10. Control and periodic acid-oxidized sections with PAS staining respectively. Scale bar = 10 µm.

Fig. 11. Transmission electron micrograph of a cell wall with PATAg reaction. Note weak reaction in fibrils and no silver deposits in amorphous material. Scale bar = 0.2 µm.

Figs 12–14. Fluorescence labeling with AMF-alginate probe with 4 (Fig. 13) and 40 (Figs 12,14) mM Ca^{2+}.

Figs 12–14. Micrographs of transverse sections of thalli.

Fig. 12. General view to show epidermal cells without fluorescence in their walls (double arrow).

Figs. 13–14. Details of cortical cells. Note increase of fluorescence in figure 14. In both cases plasmodesmata areas denote less intense labeling (arrows). Scale bars = 20 µm.

(Table 1) revealed fucoidan as the most abundant polysaccharide fraction, since alginate extraction with alkali does not significantly reduce staining at pH 4.4. It must be taken into account that fractionation of alginates and fucoidans in alkali or acid extractions is not absolute; low-molecular-weight alginates can be extracted together with fucans in acid medium (Ponce *et al.* 2003). Our previous studies allowed us to reject low sulfate content, for it can reach values above 30% in fucoidan fractions. Weaker dye binding suggested some kind of blockage of sulfate groups, preventing interactions with the cationic stain. In a similar fashion and taking into account the results of precipitation with cetrimide, Ponce *et al.* (2003) suggested that the interaction of sulfate groups (probably through calcium bridges) precluded the interaction of these anionic groups with cationic detergents.

Monosaccharide analysis of fucoidans (the major cell wall component of this seaweed) showed that glucuronic acid is absent or in trace amounts (Ponce *et al.* 2003). Thus, the weak staining observed with ruthenium red is in agreement with the scarcity of carboxylic groups in the more abundant polysaccharide of these cell walls.

Though berberine has been used as a probe for phenolic compounds in lignified or suberized cell walls (Lulai & Morgan 1992), physodes did not bind the fluorochrome. Instead, we attribute berberine fluorescence in cell walls mainly to the presence of sulfate and only to a smaller extent to carboxylate groups in fucans and alginic acid, as it has been reported for sulfated glycosaminoglycans and proteoglycans such as heparin, chondroitin sulfate and hyaluronic acid in animal tissues (Enerbaéck 1974; Stockert 2000).

Alginate and cellulose, but not fucoidan, should be PAS positive, since only the former have free vicinal hydroxyl groups. A low ratio of alginate/fucoidan in the cell walls (as suggested by TBO staining) agreed with the results obtained with this histochemical test. In *Fucus vesiculosus*, McCully (1965, 1966) mainly found alginic acid in the fibrillar, inner portion of the cell walls together with scarce cellulose fibers. PAS negativity or very feeble staining has been reported in highly crystalline cellulose fibers (O'Brien & McCully 1981). In a similar way, Ca^{2+}-coordinated polyguluronate sequences of alginate might preclude periodic acid oxidation. In these cases, a positive reaction is obtained after partially denaturing cellulose fibers or eliminating other cell wall polysaccharides in strong alkali (Jensen 1962) or by prolonged treatment with periodic acid (Krishnamurthy 1999). In our case, extending periodic acid oxidation did not significantly enhance the reaction.

Hughes & McCully (1975) and Krishnamurthy (1999) observed that alginic acid, but not sulfated fucans, binds Calcofluor White. Our results indicated certain unspecificity of fluorescent brightener binding, especially when confronting low alginate content in cell walls suggested by PAS reaction and TBO metachromasia.

More specific localization of alginate was obtained with the alginate-AMF probe. Note, for example, that no fluorescence was detected in intracellular polysaccharides. Ectocarpales lack alginate in the extracellular matrix (Larsen 1981; Craigie *et al.* 1984; Larsen *et al.* 1985), in contrast to Fucales (Vreeland & Laetsch 1989). Fluorescent probe specificity is based on its gelling interaction in presence of Ca^{2+} with polyguluronate blocks of alginate (Vreeland & Laetsch 1989). Binding strength varies with the length of cooperative binding, the frequency and distribution of mismatched segments and macromolecular environment. Several authors have reported differences in labeling or alginate composition according to plant age, size, tissue or habitat (Haug *et al.* 1974; Stockton *et al.* 1980; Craigie *et al.* 1984; Cheshire & Hallam 1985; Vreeland & Laetsch 1989). Labeling experiments in *A. utricularis* were all carried out with October specimens (spring plants) of approximately the same size. Differential binding affinity of the probe might indicate localized heterogeneities in alginate gel strength. For example, inner cortical cells might have the distribution or abundance or length of guluronate (G) blocks in alginate, allowing a higher affinity for the probe (Smidsrød & Grasdalen 1984). In contrast, cell walls in the outer layers probably have shorter G blocks.

The presence of more abundant gelling alginate subunits in the cell walls can be correlated with the mechanism of cell expansion and thallus growth. The sporophyte of *A. utricularis* is characterized by a trichothallic apical growth and development (Clayton 1985). The outer layers of the thalli are the result of lateral displacement of cells breaking off from the side walls of the apical pit. Inner medullar cells, once cut off apically, do not further divide intercallarly. Consequently, the only mode in which they can follow the growth of the saccate thalli is by elongation. This implies that cell walls in inner layers will be thinner as a consequence of expansion along the longitudinal axis. Once elongated, fibrillar pattern is stabilized by consolidating polysaccharide tertiary structures, for example, with Ca^{2+} "egg-boxes" (Rees 1977) in alginates. On the other hand, bridging by Ca^{2+} in polyguluronate occurs whether the chain is single stranded or associated (Smidsrød 1974), while periodic acid oxidation takes place in single-stranded chains. This might explain the difference in intensity between fluorescent probe binding and PAS reaction.

In conclusion, the histochemical observations of *A. utricularis* have allowed confirmation of data from exhaustive extraction of polysaccharides from the algal thalli, namely, a low alginate/fucoidan ratio in the cell wall. Alginate is located in the inner portion of cell walls. Egg-box structure of alginate in inner cortical cells may be related to the mechanism of algal growth. Abundance of fucoidan is confirmed by extraction and staining of semithin sections; weak staining of nonextracted material is in agreement with previously proposed blockage of sulfate groups in these polysaccharides.

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