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Cellulose in algal cell wall : an "*in situ*" localization

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SUMMARY

In order to ascertain the presence and the *in muro* localization of cellulose, the enzyme-gold affinity test was applied to algal cell walls. The high specificity of affinity cytochemistry allowed us, by using the enzyme cellulase, to confirm the available biochemical data and to give a map of the cellulose localization in different algal groups. Taking into account the complex skeletal polysaccharide structure and composition of the algal cell walls, this method proved to be a reliable tool in this field.

INTRODUCTION

In land plants, cellulose is the only skeletal polysaccharide, which mostly provides the tensile strenght of the cell wall. In marine algae cellulose accounts for a smaller and more variable proportion than in higher plants and in some species can be lacking. In both red and brown algae the cellulose content is rather low (Frei and Preston, 1961). Most green algae have a cellulosic wall, with cellulose content ranging up to 70% of the dry weight (as Chladophorales and Ulvales, Kloareg and Quatrano, 1988). Furthermore, algal cellulose is rarely a pure ß-1,4 glucan; more frequently it contains sugars other than glucose, commonly xylose (Mackie and Preston, 1974). Skeletal polysaccharides replacing cellulose in the algal cell walls are xylans (mainly ß-1,3 linked, McCandless,1981) and mannans (mainly ß-1,4 linked). They perform a structural function, but they have physico-chemical properties different from those of the more rigid cellulose microfibrils.

In the algal cell wall the skeletal polysaccharides are arranged in microfibrils, embedded in a rather amorphous and abundant matrix. The microfibrils present variable orientation, from parallel to disperse (Mackie and Preston, 1974). The cellulose in the cell wall can be detected and localized by ultrastructural cytochemical tests, such as lectins and periodic acid-thiosemicarbazide-silver proteinate (PATAg). The scarce specificity of this latter test (the response is positive for many kinds of carbohydrates, Roland and Vian, 1991) and the heterogeneity of the algal wall components lower the significance of the positive responses.

In a previous paper (Baldan *et al.*, 1995) the enzyme-gold labelling proved to be a reliable tool for a precise identification and localization of cellulose in the cell wall of *Porphyra leucosticta* Thur. (Bangiaceae, Rhodophyceae) during the life cycle. This method, introduced by Bendayan (1984), uses the cellulase enzyme complex (conjugated with gold particles as electron-dense

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markers) as a cytochemical probe for *in situ* localization of cellulose.

Here we apply this affinity technique to identify and localize cellulose in the cell wall of some red, brown and green algae. In order to compare the reliability of the two methods, the PATAg test was also routinely used.

MATERIALS AND METHODS

Thalli of *Gracilaria verrucosa*(Hudson)Papenfuss (Gracilariaceae, Gigartinales, Rodophyceae), *Dictyota dichotoma*, (Hudson) Lamour. (Dictyotaceae, Dictyotales, Phaeophyceae), *Fucus virsoides* J. Agardh (Fucaceae, Fucales, Phaeophyceae), *Cystoseira barbata* var. *tophuloidea* (Erceg.) Giaccone (Cystoseiraceae, Fucales, Phaeophyceae), *Ulva laetevirens* Areschoug (Ulvaceae, Ulvales, Chlorophyceae), *Enteromorpha intestinalis* (L.) Nees (Ulvaceae, Ulvales, Chlorophyta) and *Chaetomorpha aerea* (Dillwin) Kutzing (Cladophoraceae, Cladophorales, Chlorophyta) were collected in Adriatic Sea (Chioggia, Venezia, Italy). *Halimeda tuna* (Ellis and Solander) Lamour. (Udotaceae, Siphonales, Chlorophyceae) and *Flabellia petiolata* (Turra) Nizamuddin (Bryopsidales, Chlorophyceae) came from Tirreno Sea (Ischia, Napoli, Italy).

For conventional trasmission electron microscopy, samples of thalli were processed according to Mariani *et al.*, 1985. For polysaccharides localization periodic acid-thiosemicarbazide-silver proteinate (PATAg) test was applied to ultrathin sections. Controls were carried out omitting the thiosemicarbazide reaction or without periodic acid oxidation.

For the enzyme-colloidal gold labelling, colloidal gold suspension was prepared according to Frens (1973). Ultrathin sections were collected on grids and floated on a drop of the enzyme gold complex containing solution prepared according to Berg *et al.* (1988); we used a commercial cellulase (EC3.2.1.91, CEL, Worthington). Controls were performed incubating grids in a enzyme-gold complex and carboxymethyl-cellulose (1:1 v/v) containing solution.

RESULTS AND DISCUSSION

When cells at the thallus surface of *Gracilaria verrucosa* were examined under electron microscope after the cellulase-gold test, a rather dense labelling appeared distributed mainly in the immediate wall (*sensu* Kloareg and Quatrano, 1988). Only scarce gold granules were randomly dispersed in the outer zone of the cell wall which looked predominantly amorphous (Fig. 1a). The PATAg test confirmed the distribution of positive polysaccharides mainly in the microfibril-rich region proximal to the plasma membrane (Fig. 1b). The ultrastructural organization of *Gracilaria* cell wall has been previously investigated before and after extraction of the agar matrix polysaccharides, but the composition of the microfibrils has not been clearly cytochemically elucidated (Verdus *et al.*, 1986). Here we unambiguously show the presence of cellulose in *Gracilaria*, and its preferential localization in the inner part of the external cell wall. The same cellulose distribution was also observed inside the thallus, where no labelling was present in addition to the microfibrillar region of the proper cell wall (data not shown). Among Phaeophyceae, the cellulose localization was examined in the thalli of *Dictyota dichotoma*, *Fucus virsoides* and *Cystoseira barbata* var. *tophuloidea*. Owing to the similarity of their cell wall composition, all the examined species showed the same behaviour with regard to the two cytochemical tests we used.

The microfibrils were markedly labelled with the PATAg test, being uniformly covered by fine silver proteinate granules (Fig. 1c). With cellulase-gold complex, a specific label was present, but not so extensive. Gold precipitates lined short regions over some microfibrils (Fig. 1d). These results well match the known chemical composition of the cell wall microfibrillar fraction, also confirming the high reliability of the *in situ* affinity test. Besides being present in the Phaeophyceae cell walls at a low level, cellulose can be masked by insoluble Ca^{2+}-alginates, which are thought to form a network around the crystalline cellulose (Kloareg and Quatrano, 1988). Whereas alginates, as well as cellulose, can be labelled by the PATAg test (Mariani *et al.*, 1985), thus accounting for the intense positive responsiveness observed in the cell wall, they cannot be recognized by the more selective cellulase-gold procedure. It is noteworthy that inside the thallus the large intercellular spaces are filled with a strong PATAg-positive thin fibrillar material, which is negative to the cellulase-gold test (not shown).

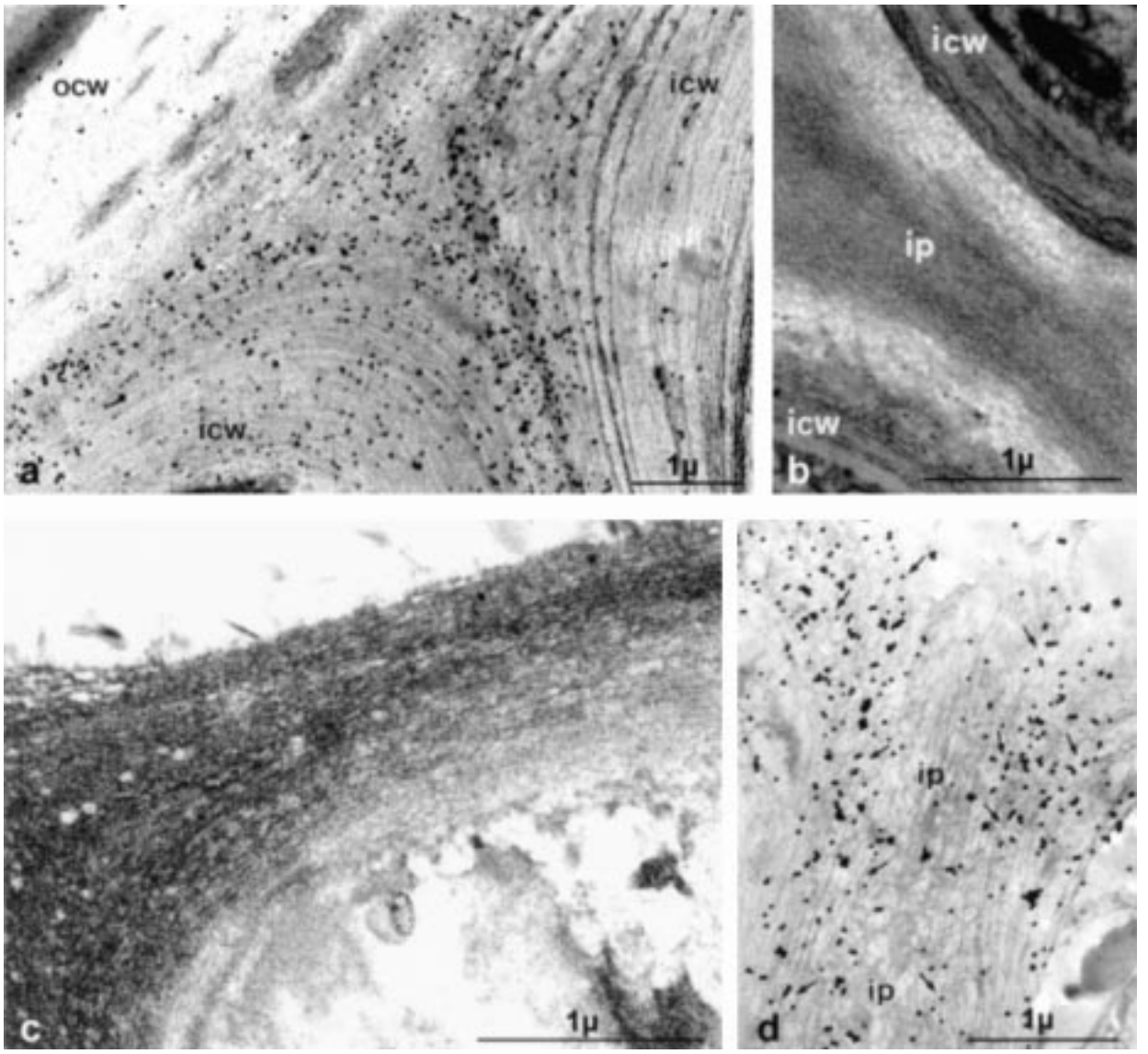

Fig. 1 - Cellulose localization in Rhodophyceae and Phaeophyceae. **a, b:** *Gracilaria verrucosa*. Gold granules are distributed mainly in the inner layer of the cell walls (icw) which are in close contact together (1a) ; the inner layers of the cell walls (icw) are strongly positive also to the PATAg test (1b). **c, d:** *Cystoseira barbata*. The silver deposits (PATAg test) are uniformely spread on the whole wall (1c); gold granules are more abundant over the cell wall microfibrils (arrows) than over the intercellular matrix (1d). ip= intercellular polysaccharides; ocw= outer cell wall.

In the green algae, the chemical composition of the skeletal fraction varies greatly, and cellulose can be present in different amount and/or with different degrees of pureness and organization, or it is lacking (Painter, 1983). In this last case, the function of the cellulose is taken over by other skeletal polysaccharides like crystalline beta-1,3 xylans. The species we chose are representative of different situations: algae with true very crystalline cellulose in crossed fibrillar texture (*Chaetomorpha aerea*), with less ordered cellulose (*Ulva laete-virens, Enteromorpha intestinalis*) and without cellulose (*Halimeda tuna, Flabellia petiolata*). In all the organisms the skeletal polysaccharides were organized in microfibrils, clearly evident under electron microscope. *Chaetomorpha* showed the most regular arrangement, with microfibrils lying down parallel to each other in

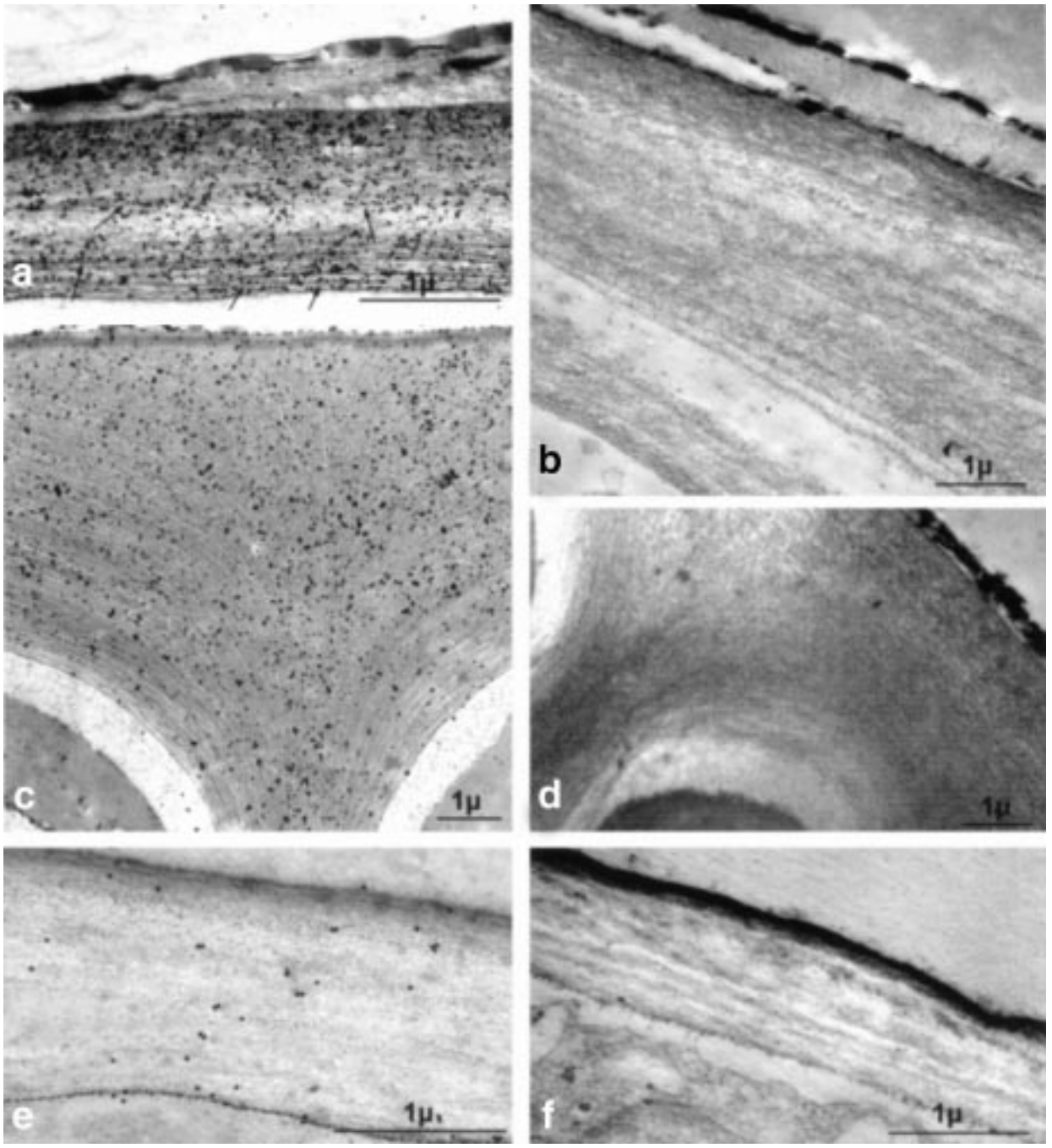

Fig. 2 - Cellulose localization in Chlorophyceae. **a, b:** *Chaetomorpha aerea*. Gold particles are densely distributed and often aligned (arrows) over microfibrils which lie down parallel to each other in lamellae (2a); PATAg test is strongly positive (2b). **c, d:** *Ulva rigida*. With both the cellulase-gold (2c) and the PATAg tests (2d), the cell wall appears marked by an uniform but rather weak label. **e, f:** *Halimeda tuna*. No labeling is observed with either the cellulase-gold (2e) or the PATAg (2f) tests.

lamellae. From one lamella to the next one the microfibrils shifted their orientation, so that the cell wall resulted with helicoidal arrangement (evident in Fig. 2a). In *Ulva and Enteromorpha*, the microfibrils in the lamellae appeared with more loose texture. The microfibrillar organization in

Halimeda and Flabellia was not evident, so that the cell walls looked like an homogeneous, scarcely electron-dense cell boundary (Mariani, 1978).

The responsiveness to the cytochemical tests reflects the different situation of the cell wall composition and organization showed by the examined species. In *Chaetomorpha* both tests were highly positive. The silver proteinate deposits appeared aligned over the microfibrils along the entire cell wall thickness (Fig. 2b). The gold particles were also abundant, and underlined the trend of the skeletal polysaccharides (Fig. 2a). These strong labels are in agreement with the high cellulose level and purity (Mackie and Preston, 1974) and with the X-ray diffraction pattern observed in this species (Mc Candless, 1981).

The *Ulva* and *Enteromorpha* cell wall was more weakly labelled by both tests (Figs. 2c and 2d). In particular, the gold particles were distributed over the entire cell wall (Fig. 2c). The more loose fibrillar texture allowed the visualization of the gold electron-dense granule regular arrangement over long tracts of microfibrils. The rather weak labelling, in spite of the high level of cellulose, could be ascribed to masking of cellulose by its close association in the cell wall with xylans and β-1,3 glucans (Dodson and Aronson, 1978; Mc Candless, 1981).

The third group of algae we considered (siphonous green algae) is known to have xylans as the main skeletal polymer, essentially with β-1,3 linked xylose units (Mackie and Preston, 1974; Percival, 1979; Kloareg and Quatrano, 1988). As evident in Figs. 2e and 2f, no true labelling was found with either the cytochemical tests. The lack of responsiveness to both tests depends on their lack of specificity towards the chemical component of the microfibrils. Because of the β-1,3 linkage present in the skeletal xylans, the PATAg test is unable to visualize the polymer.

Furthermore, because of the strict specificity of an enzyme to its substrate, which is at the basis of the enzyme-gold cytochemical approach, obviously the cellulase cannot be successfully applied to label polymers other than cellulose. All the controls were negative: no labelling was observed when carboxymethylcellulose was added to the gold-enzyme complex .

CONCLUSIONS

We can examine the results presented in this paper, which are based on the already known biochemical composition of the algal cell wall and the basic features of its ultrastructural organization, by considering the advantages and the limits of the enzyme-gold cytochemistry. First of all, the specificity provided by cellulase-gold test due to the affinity of the enzyme versus its substrate makes it a very reliable tool for stating the absence or presence of cellulose, even in small amount. The lack of responsiveness of *Halimeda* and *Flabellia* parietal polysaccharides or the weak labelling of the brown algae cell walls seem to be good exemplifications: not all the microfibrils we see at the ultrastructural level are indeed made of cellulose. Furthermore, the distribution of the labelling inside a single wall or a thallus region provides, without induced alterations, a map of the cellulose localization. This can supply unexpected details on the biochemical organization of the algal cell wall. In *Gracilaria* we noted a preferential distribution of the gold particles in the fibrillar zone of the walls. In *Cystoseira*, the large intercellular spaces filled with thin microfibrils are almost devoid of labelling. Thus, in this zone the structural role is clearly fulfilled by polysaccharides other than cellulose (Mariani *et al.*, 1985).

The biochemical complexity of the algal cell wall composition, the possible association of cellulose with other polymers, the variable degree of crystallinity found in the algal cellulose can be reasons for a limited accessibility of the enzyme to its substrate, thus lowering the response to the test. For example, the labelling of the cell wall in *Enteromorpha* and *Ulva* could illustrate this notion. Well programmed chemical treatments eliminating matrix or adsorbed polysaccharides could help exposing cellulose and increasing the binding of the enzyme to its target molecule.

The limited availability of highly purified and appropriate enzymes, essential for the success and reliability of the *in situ* affinity technique, narrows the field of the possible applications of this test mainly to algal studies. Nevertheless, when compared with the frequently strong, uniform and scarcely selective staining done by the PATAg reaction, the results obtained with cellulase-gold, even if to be considered with caution, can add further new and useful information to the background of our knowledge of the algal wall organization.

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