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Presence of a Nonhydrolyzable Biopolymer in the Cell Wall of Vegetative Cells and Astaxanthin-Rich Cysts of *Haematococcus pluvialis* (Chlorophyceae)

Anton Montsant, Aliza Zarka, and Sammy Boussiba*

Microalgal Biotechnology Laboratory, Albert Katz Department of Dryland Biotechnologies, Jacob Blaustein Institute for Desert Research, Ben-Gurion University of the Negev, Sede-Boqer Campus, Sede-Boqer 84990, Israel

Abstract: The green alga *Haematococcus pluvialis* accumulates massive amounts of the red pigment astaxanthin in response to stimuli inducing it to form cysts. During the encystment process the cell wall undergoes a clear hardening and thickening. In this work, a cell wall fraction withstanding successive acid and basic hydrolysis was isolated and proves to be algaenan by Fourier transform infrared spectroscopy. This compound is equally abundant in nonmotile vegetative cells and astaxanthin-rich cysts. This finding indicates that the synthesis of algaenan does not require the activation of the machinery for the massive production of secondary carotenoids. We conclude that algaenan cannot cause the changes occurring in the cell wall during the encystment process without the involvement of other cell wall components.

Key words: algaenan, astaxanthin, cell wall, *Haematococcus,* secondary carotenoids, sporopollenin.

INTRODUCTION

The green alga *Haematococcus pluvialis* has recently been identified as a promising source of the red ketocarotenoid astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione), widely used as a feed supplement in aquaculture and poultry for coloring fish flesh and egg yolk (Lorenz and Cysewski, 2000). Massive production of this pigment occurs under several stress conditions, such as a high salt concentration or deprivation of nutrients, e.g., phosphate or nitrogen (Boussiba et al., 1992). Under such conditions, cell division ceases and cells increase in size and accumulate astaxanthin esters in cytoplasmic oil drops (for a recent review, see Boussiba, 2000). The formation of astaxanthin-rich cysts is also accompanied by the development of a thick cell wall (Mesquita and Santos, 1984).

No information on the composition of the cell wall of *H. pluvialis* cysts has been given so far. However, in other microalgae accumulating secondary carotenoids (SC), a complex cell wall polymer that is resistant to several chemical and enzymatic treatments, termed algaenan, has been described (Atkinson et al., 1972; Burczyk, 1987a). This compound is thought to be responsible for the recalcitrance of *H. pluvialis* cysts to physical disruption and enzymatic degradation (Lee, 1999). In some chemical properties, such as resistance to nonoxidative aggressive reagents and infrared absorbance profile, algaenan is very similar to pollen sporopollenin, which until recently was considered to be biosynthesized through polymerization of carotenoids

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*Corresponding author: Prof. Sammy Boussiba, telephone 972-8-6596795; fax 972-8-6596802; e-mail: sammy@bgumail.bgu.ac.il

(Brooks and Shaw, 1968). Furthermore, for a number of years after microalgal sporopollenin-like compounds were first reported (Atkinson et al., 1972), all the species in which these materials had been reported also possessed SC. These 2 facts led to the incorrect assumption that algaenan had a carotenoid-related origin (Atkinson et al., 1972). At present, some species possessing chemically resistant cell wall layers and no SC are known (Puel et al., 1987), and the noncarotenogenic composition of algaenan and sporopollenin has been demonstrated (Derenne et al., 1992; Blokker et al., 1998; Dominguez et al., 1999). Nevertheless, it seems that there is a link between SC and algaenan that remains unexplained. Some inhibitors of the carotenogenesis also block the formation of sporopollenin-like cell wall polymers (Burczyk, 1987b). In addition, some mutants of SC-accumulating species lacking the ability to accumulate carotenoids also lack the resistant cell wall layer present in their wild types (Burczyk, 1987a).

Since we have been interested in factors controlling the rate of astaxanthin accumulation in *H. pluvialis* (Boussiba, 2000), it was important to clarify the interrelationships between wall formation and pigment accumulation, the 2 major events occurring during the encystment process. Toward this goal, we have started to characterize the cell wall composition in this alga. In this study we report for the first time the presence of algaenan in the cell wall of vegetative and astaxanthin-rich encysted cells.

Materials and Methods

Strain

Haematococcus pluvialis Flotow (Chlorophyceae, order Volvocales) was obtained from the Scandinavian Culture Center for Algae and Protozoa (SCCAP) at the University of Copenhagen, Denmark.

Culture Conditions

Vegetative cells were cultured in BG_{11} medium as modified by Boussiba and Vonshak (1991). The temperature was maintained at 25°C, light was supplied continuously at a photon flux density of 100 μmol $m^{-2}s^{-1}$, and aeration was provided by bubbling air containing 1.5% CO_2. Under these conditions the pH of the culture fluctuated between 6.8 and 7.5 and doubling time was 13 hours. Encystment was induced by resuspending vegetative cells at the exponential growth phase in nitrogen-free modified BG_{11} medium for 5 days.

Hereafter the term "vegetative cells" represents cells found in a culture of *H. pluvialis* at the exponential growth phase. Under the growth conditions used in this study, the majority of the cells are round and nonflagellated because the motile stage only lasts for a few hours after the zoospores are released from the mother cell. The encysted astaxanthin-rich cells, which are not dividing, are referred to as "red cysts".

Growth Parameters and Cell Characterization

Cell number and chlorophyll and total carotenoid content were determined as previously described (Boussiba and Vonshak, 1991). Cell size was measured by means of a ruler fitted in the objective of the microscope.

Isolation of Cell Walls

Samples containing 300 to 800 × 10^6 vegetative or encysted algal cells were harvested, resuspended in cold HEPES (*N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid]) buffer, pH 7, and passed through a French press cell (P > 20,000 psi) twice. Broken cells were rinsed with 300 ml of cold HEPES buffer (×3) to remove cytoplasmic matter. The pellet was extracted with 95% dimethylsulfoxide (DMSO) at 70°C to remove pigments and most of the starch grains until 2300 *g* centrifugation yielded a noncolored supernatant (pellets almost white).

Isolation of Nonhydrolyzable Material

The procedure used here is a modification of a previously described method aimed at minimizing condensation reactions between sugars and proteins (Allard et al., 1998). It consisted of 6 steps. (1) *Removal of loosely bound lipids* by extraction for 2 hours at 40°C with each of 3 solvents: MeOH, MeOH-CH_2Cl_2 1:1 (vol/vol) and CH_2Cl_2-hexane 1:1 (vol/vol). (2) *Enzymatic degradation of cellulose* was by digestion with cellulase; enzyme-substrate ratio of 2.15 U cellulase (Sigma) per 10^6 cell walls (35°C, 3 hours). (3) *Reduction of polysaccharide content* was by treatment with aqueous TFA solutions of increasing normality (2, 4, 6 N for 3, 14, 10 hours, respectively) at 90°C to prevent the formation of melanoidin-like contaminants. (4) *Removal of tightly bound lipids* by saponification with 1 M KOH in 96% MeOH (70°C, 1 hour). (5) *Removal of carbohydrates and proteins* was by acid hydrolysis with 6 N HCl (95°C, 20

hours). (6) *Additional saponification* conducted in order to release the components directly esterified to the nonhydrolyzable fraction (70°C, 1 hour). All treatments were carried out in 30-ml screw-cap tubes with 5 ml of reagent and isolated pigment-free cell walls as the starting material. The absence of cytoplasmic components decreased the probability of formation of contaminants. Between steps the pellets were washed by suspension in 25 ml of water (after treatments in aqueous solution) or of MeOH (after reactions in organic medium). Remaining residues were always recovered by 2300 *g* centrifugation for 5 minutes in steps 1 and 2, and from then on by 10,000 *g* centrifugation for 5 to 20 minutes.

Preparation of Acetolysis-Resistant Material

Culture samples containing 100×10^6 cells were pelleted and extracted with DMSO. The pellet was washed with glacial acetic acid and resuspended in 3 ml of 10% sulfuric acid in acetic anhydride (vol/vol). After 30 minutes of digestion in a boiling water bath, the resulting material was pelleted and washed several times with glacial acetic acid and with water.

Light and Fluorescence Microscopy

Samples were observed with a Zeiss Axioskop fitted with a high-pressure mercury lamp (HBO 50 W) and a Zeiss MC80 microscope camera. ARM fluorescence was induced by a filter allowing maximum excitation wavelength at 365 nm and viewed through a 390-nm cutting-off filter.

Electron Microscopy

Cells were fixed in the growth medium using super fixer (2% glutaraldehyde, 3% paraformaldehyde, 1% acrolein, and 1% DMSO) in 0.1 M cacodylate buffer, pH 7, at room temperature. The next day, cells were postfixed with 1% OsO_4, washed, and stained with 2% uranylacetate and examined with a Philips EM-410 100 kW transmission electron microscope.

Spectroscopy

Potassium bromide pellets containing 1% of freeze-dried nonhydrolyzable or acetolysis-resistant material (wt/wt) were prepared and their Fourier transform infrared (FTIR) spectra were obtained with an Impact 410 infrared spectrometer.

Results

Considerable changes in the physical properties of the cell wall occurring during encystment were identified. The walls of vegetative cells and of red cysts were found morphologically different by microscopic observations of cell wall homogenates obtained by a high-pressure breakage. While the disruption of vegetative cells resulted in an oval cell wall (Figure 1, A), the walls of red cysts maintained a round shape (Figure 1, B). This difference may reflect a difference in mechanical strength. This assumption was confirmed by the different pressures required for breaking the vegetative cells and the red cysts. At a moderate pressure (4000 to 10,000 psi), the vegetative cells were highly susceptible to physical disruption, while the red cysts were leaky but remained almost intact (i.e., vegetative cells burst completely but red cysts only suffer some fissures). Only when a very high pressure was applied (20,000 psi) could red cysts be completely disrupted (not shown). In agreement with this finding, the cell wall of the red cysts was remarkably thicker (2–3-fold) than that of the vegetative cells in transmission electron micrographs (Figure 2, B vs. A).

The possible involvement of algaenan in the cell wall changes occurring during encystment was investigated. The most appropriate method reported so far for isolating highly pure algaenan (Allard et al., 1998) is complex and time-consuming. Thus we first submitted vegetative and encysted cells to acetolysis, which although it was shown to be inadequate for isolating pure unmodified sporopollenin-like polymers (Berkaloff et al., 1983), is useful for detecting the possible presence of a refractory compound rapidly. Microscopy observations of pigment-free *H. pluvialis* cells after acetolysis showed that both vegetative cells and red cysts possess an acetolysis-resistant fraction in the cell wall (Figure 3, A and B). This fraction showed an intense autofluorescence when excited with ultraviolet light (Figure 3, C and D), a common feature of sporopollenin-like materials derived from various biological origins (Puel et al., 1987; Van Winkle–Swift and Rickoll, 1997; Talyzina, 1998; Roschina and Karnaukhov, 1999). No acetolysis-resistant material was obtained from the vegetative flagellated stage—not surprisingly, since they exhibit a gelatinous matrix rather than a cell wall around the protoplast (not shown).

An elaborate method based on that performed by Allard et al. (1998) (see Materials and Methods) was employed in order to isolate a highly pure unmodified nonhydrolyzable material and proceed to examine its chemical

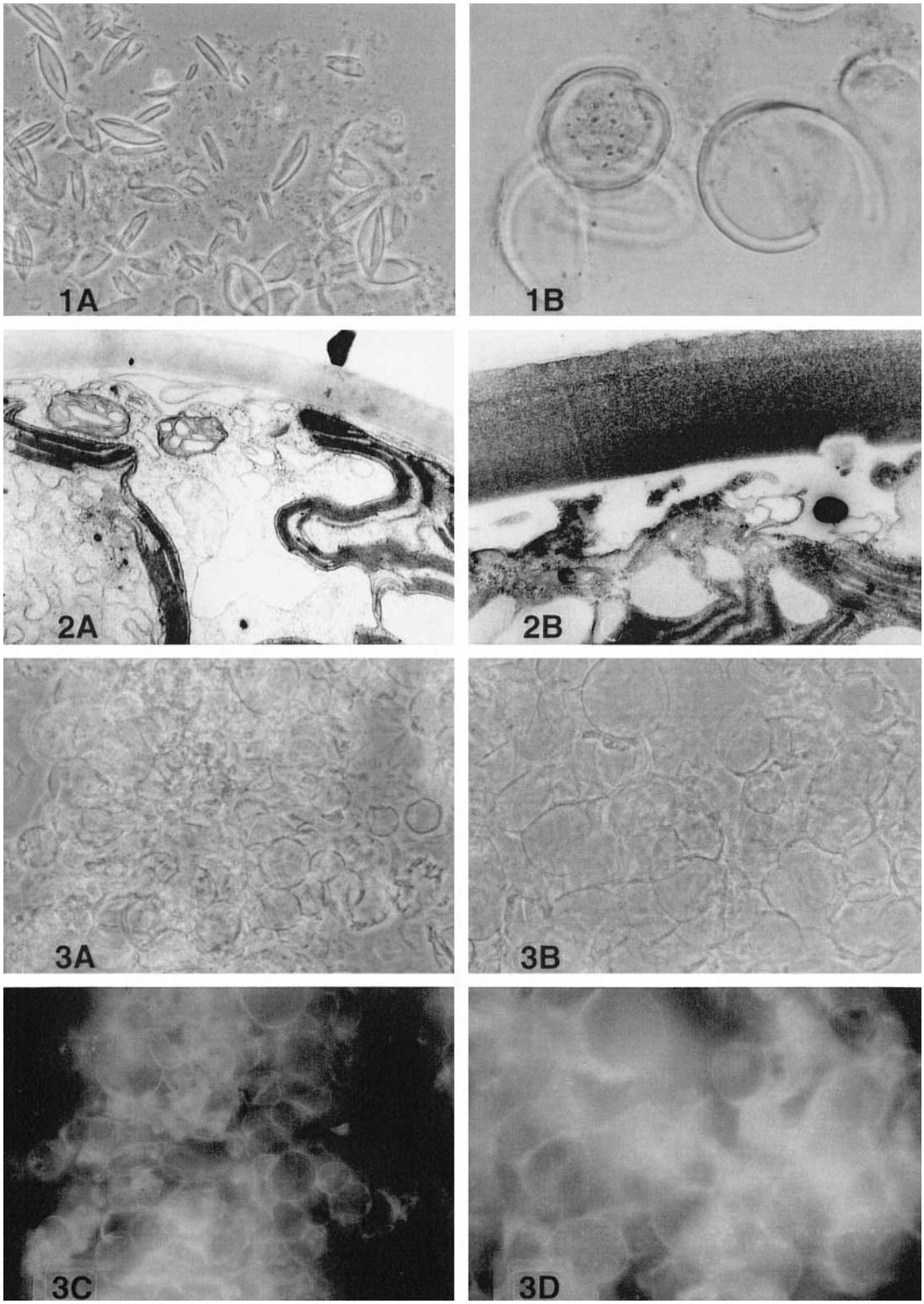

Figure 1. Isolated cell walls from vegetative cells (**A**) and red cysts (**B**). Magnification: × 370.

Figure 2. Transmission electron micrographs of the cell wall of a vegetative cell (**A**) and of a red cyst (**B**). Magnification: × 13,000.

Figure 3. Acetolysis-resistant materials obtained from vegetative cells (**A**) and from red cysts (**B**) visualized by light microscope and their UV-induced autofluorescence (**C** and **D,** respectively). Magnification: × 215.

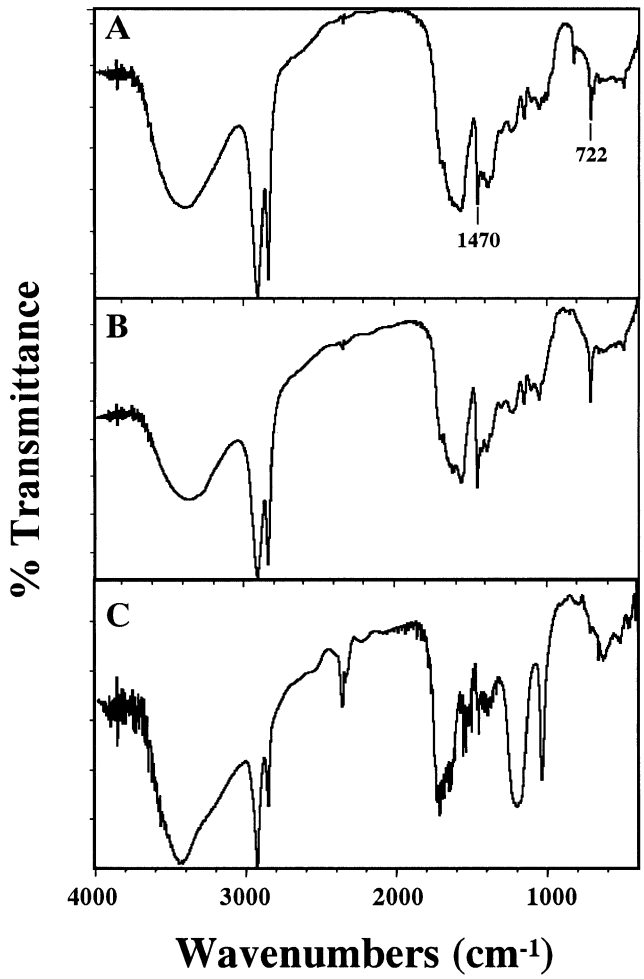

Figure 4. FTIR spectra of nonhydrolyzable materials (NHM) derived from red cysts (**A**) and from vegetative cells (**B**) and of acetolysis-resistant materials (ARM) derived from vegetative cells (**C**).

nature by means of infrared spectroscopy. The characteristics of the samples used in this assay are shown in Table 1. The FTIR spectra presented in Figure 4 indicate that the nonhydrolyzable material from *H. pluvialis* is highly saturated, as shown by the sharp peaks around 2900 cm^{-1} (CH_3 and CH_2 stretching), the peak around 1470 cm^{-1} (CH_3 and CH_2 asymmetric bending) and the signal at 722 cm^{-1} (skeletal vibrations of polymethylenic chains). The presence of hydroxyl groups is reflected in the absorption band centered at 3500 cm^{-1}. The band in the region 1730 to 1600 cm^{-1} may be due to double bond stretching or to carbonyl, possibly indicating melanoidin-like contaminants (Allard et al., 1998). It is noteworthy that the fingerprint region (1400–800 cm^{-1}) is identical in the FTIR spectra of the nonhydrolyzable material from both vegetative cells and red

Table 1. Pigment Content, Cell Dry Weight, and Cell Size of Vegetative Cells and Red Cysts

Measurement	Vegetative cells	Red cysts
Mean cell diameter* (μm)	20	55
Dry weight (ng/cell)	1.33	8.1
Chlorophyll (pg/cell)	29.14	47.25
Carotenoids (pg/cell)	6.12	241
Carotenoids:Chlorophyll	0.21	5.1

*Since the cultures were not grown syncronously, the cell size is heterogeneous, varying between 15 and 24 μm for the vegetative cells and 40 and 70 μm for the cysts.

cysts (Figure 4, A vs. B). Since it is virtually impossible for 2 different molecules to display the same fingerprint because of the high density of signals in this regimen, the NHMs isolated from cells at the 2 stages are the same compound. The amount of NHM was found to be 2.2 ± 0.08 pg/cell ($n = 4$) at the vegetative stage (i.e., 0.15% of the cell dry weight) and 17 ± 0.7 pg/cell ($n = 4$) at the encysted stage (0.2% of the cell dry weight).

Our spectra also reflect that acetolysis of DMSO-extracted cells yields a different residue (Figure 4, C vs. B), owing to the partial acetylation of the acetolysis-resistant components (i.e., modification of their chemical structure) and to the formation of contaminants. The alteration of the band at 3500 cm^{-1} and the increased intensities of the signals around 1700 cm^{-1} (C=O) and 1200 to 1000 cm^{-1} (C–O–C) are mainly due to the formation of acetic acid esters in the positions of the hydroxyl groups.

Observation of acetolysis-resistant material and nonhydrolyzable material under light microscope revealed that the morphology of the residues obtained by the 2 methods also differed remarkably. While acetolysis of whole cells yields continuous cell-wall remains with their native spherical arrangement (Figure 3), successive acid and basic hydrolysis of whole cells results in a disaggregated residue composed of small granular flakes (<5 μm) with a faint autofluorescence (not shown).

Discussion

The FTIR absorbance profiles presented here provide solid evidence that the nonhydrolyzable compound detected in *H. pluvialis* cell walls is the microalgal sporopollenin-like polymer termed algaenan. When the spectra presented in

Figure 4, A and B, are compared with spectra found in the literature, the nonhydrolyzable material of *H. pluvialis* appears very similar to the algaenans isolated from *Botryococcus braunii, Tetraedron minimum, Pediastrum boryanum,* and several species of the genera *Scenedesmus* and *Chlorella* (Allard et al. 1998; Blokker et al. 1998; Allard and Templier, 2000) and to pollen sporopollenin from 4 different plant species (Dominguez et al., 1999). Dicarboxylic acids, alcohols, and fatty acids with an average chain length between C_{22} and C_{26} have been proposed as building blocks of the algaenan isolated from *Chlamydomonas monoica* (Blokker et al., 1999), a close relative of *H. pluvialis.* The linkages between these monomers would be ether and ester bonds, which may be represented in the spectra of *H. pluvialis* algaenan by the peaks around 1100 cm^{-1}.

The changes occurring in the cell wall of *H. pluvialis* during its encystment are not related to the onset of the deposition of algaenan, because this material was also found in cells at early vegetative stages. Our results further indicate that the described cell wall changes cannot be caused by a higher abundance of the sporopollenin-like polymer at the encysted stage. The algaenan content per cell is around 8 times higher in the cyst than in the vegetative cell. Nevertheless, since the outer surface of the cell (i.e., cell wall area) increases linearly with its squared radius, it emerges from the average cell sizes shown in Table 1 that the cell wall area is also increased by a factor of 8 during encystment. The algaenan areal density in the cell wall at the 2 stages is thus the same. Consequently, this compound can not contribute to the observed cell wall thickening, and could hardly account for the other changes on its own without the participation of other cell wall components.

We cannot exclude the involvement of algaenan in some of the described changes, since the organization of the algaenan moieties may change during encystment. In young cells of *Chlorella fusca,* discrete plaques of a sporopollenin-like material were observed to extend at their edges during the cell growth until all the plaques bound together to form a continuous spherical surface (Atkinson et al., 1972). In *H. pluvialis,* algaenan does not seem to form a continuous layer at either stage, as the most accurate procedure (successive acid-basic hydrolysis) always yielded a fragmented residue. A change in the assemblage of algaenan with other cell wall components (e.g., a different degree of cross-linking between algaenan and polysaccharides) could be responsible for some of the differences observed between the cell wall of the vegetative cells and the cell wall of the red cysts.

Our results do not support any direct relationship between algaenan and SC biosynthesis. Whereas vegetative cells have a carotenoids-chlorophyll ratio of 0.21 and possess mainly β-carotene, lutein, and cantaxanthin, with only trace amounts of astaxanthin, fully developed cysts (induced by nitrogen starvation) have a carotenoids-chlorophyll ratio of 5.1 and possess astaxanthin esters as almost the sole carotenoid, i.e., 99% of the total carotenoids (Boussiba et al., 1999). If algaenan is present before the SC accumulation starts and its relative abundance remains unaffected during the massive SC accumulation, nothing indicates that SC synthesis may induce or stimulate algaenan synthesis.

The capabilities to accumulate SC and to synthesize algaenan occur together in many species and are suppressed together by certain mutations and inhibitors, as observed by Atkinson et al. (1972) and Burczyk (1987b). This phenomenon might be explained by the fundamental role of fatty acids in these 2 metabolic activities. On the one hand, as stated above, fatty acids, their derivatives, or their precursors are probably the monomeric units of several algaenans (Blokker et al., 1998, 1999) and pollen sporopollenins (Dominguez et al., 1999). On the other hand, acylglycerol droplets are necessary as a sink for the end product of the secondary carotenoid biosynthetic pathway. This requirement has been clearly proved in the β-carotene-accumulating green alga *Dunaliella bardawil,* in which inhibition of triacylglycerol synthesis also blocked the accumulation of the final carotenoid (Rabbani et al., 1998). Hence, it is possible that the building blocks for the synthesis of algaenan and the components of the apolar carotenoid-sequestering structures are closely related (e.g., have a common precursor). In fact, radioactivity from exogenous ^{14}C-labeled oleic acid is incorporated into the cell wall acetolysis-resistant fraction in *Scenedesmus communis* (Coudercher et al., 1996), and the same fatty acid has been proposed as the main component of the carotene-containing plastidic lipid globules in *Dunaliella salina* (Mendoza et al., 1999).

The identification of algaenan in the cell wall of *H. pluvialis* is reported here for the first time. The fact that this compound was found to be equally abundant in cells at both vegetative and encysted stages supports its noncarotenogenic nature. Elucidating the components that are responsible for the changes occurring in *H. pluvialis* cell wall during encystment will require further investigation.

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