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CELL WALL NEUTRAL SUGAR COMPOSITION OF CHLOROCOCCALEAN ALGAE FORMING AND NOT FORMING ACETOLYSIS RESISTANT BIOPOLYMER

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Key Word Index—*Chlorococcales*; green algae; cell wall (CW) components; neutral sugars; ketocarotenoids; acetolysis resistant biopolymer (ARB); CW-mutants.

Abstract—Algal strains belonging to the genus *Chlorococcales* differ in cell wall (CW) composition. Two types of ultrastructure for the outer cell wall layer are known. The first (pink coloured) has a trilaminar structure and contains an acetolysis-resistant biopolymer (ARB) and ketocarotenoids. The second (white) contains none of the above components. Differences in the cell wall neutral sugar composition of various strains and their relationship with the occurrence of ARB are presented. The results also show differences in the sugar pattern of cell walls isolated from homogenates (CWH) and maternal cell walls (CWM) accumulated in medium as a result of autospore liberation.

INTRODUCTION

In the last few years, two types of cell wall ultrastructure have been distinguished in algal strains belonging to chlorococcales (*Chlorophyceae*) [1–4]. The characteristic feature of the first type is the homogenous structure of the outer cell wall (CW) layer. By contrast, algae of the second type show a membrane-like, trilaminar structure (TLS) for this layer. The presence of a TLS was found in algae forming a biopolymer which was unusually resistant to drastic non-oxidative chemical treatment. In previous publications this polymer (ARB) was called sporopollenin [1–3]. The biopolymer is probably restricted to the TLS where it occurs side by side with ketocarotenoids [4–7]. The close connection between the occurrence of ARB, TLS and ketocarotenoids, as well as their absence was called the three-way-correlation [1]. According to this hypothesis carotenoids are biogenetic precursors of SP-synthesis. However, further progress in this field by the application of GC-MS, pyrolysis-GC, solid state ^{13}C NMR, FTIR and solid state ^{13}C NMR indicates a complete lack of any relationship between the TLS biopolymers and carotenoids [8–16]. The lack of such a relationship is supported by the finding that the apochlorotic alga *Prototheca wickerhami* contains ARB but does not form carotenoids [17]. According to the latter study the resistant biopolymer of *P. wickerhamii*, in common with the carotenoids, is probably a terpenoid. *Prototheca wickerhamii* is considered by some to be an apochlorotic form of chlorella [1], but this view has been questioned [18].

A number of recent studies of unicellular algae, e.g. *Botryococcus braunii* [19, 20] indicate the existence of structural differences in the composition of the free hydrocarbons and hydrocarbon-biopolymer components of the TLS of various strains of *B. braunii* [11]. The A-race produce odd carbon numbered hydrocarbons, i.e. C_{23} to C_{31}, dienic- and trienic normal hydrocarbons, while the B-race form highly unsaturated triterpenoids C_nH_{2n-10} (n = 30–37) and hydrocarbons termed botryococcenes. The L-race produces only one C_{40} isoprenoid hydrocarbon i.e. lycopa-14(*E*), 18(*E*)-diene [10, 16]. As stressed here, the L-race of *B. braunii* produces the macromolecular structure of ARB from such C_{40} isoprenoid chains. In contrast, the biopolymer of the A-race of this strain is composed of polymethylene chains and no contribution from isoprenoid moieties has been found. This also seems to be the case with the alga *Scenedesmus obliquus* which on pyrolysis was found to produce from ARB a series of long chain *n*-alkanes and *n*-alkenes [21]. It seems possible, therefore, that the class of ARB comprises biopolymers of similar resistance but different chemical nature for various algae.

All recently investigated natural strains of *Chlorococcales*, as well as UV-induced mutants lacking ketocarotenoids do not form ARB and TLS [3, 4]. Such mutants have been isolated from a parental strain of *Chlorella fusca* (C.1.1.10) which forms TLS, ARB and ketocarotenoids. Algae not forming ARB are characterized by having an homogenous, carotenoid-free outer CW-layer.

Reports in the literature concerning the sugar composition of cell walls isolated from homogenates of algal cells

Dedicated to Prof. Dr. F.-C. Czygan on the occasion of his sixtieth birthday.

(CWH) have disregarded the occurence of ARB [22–24]. Two essential patterns of neutral sugars have been discerned in CWH of strains belonging to *Chlorococcales*. The first type is characterized by the prevalence of mannose and glucose in the sugar moieties of this fraction [22]. The second type shows a prepronderance of rhamnose and galactose moieties.

This paper reports on the relationship between CW-neutral sugar composition and the presence or absence of ARB. In addition, a comparison has been made of the sugar composition of whole CWH with that of cell walls accumulated in the medium, i.e. so-called maternal cell walls (CWM), CWM corresponds to TLS [25], i.e. the residue of CWH resistant to autolytic enzymes [26, 27].

CWM accumulate in the medium during growth of algal cells which form ARB and also in a few strains which do not contain ARB in their CW (J. Burczyk, unpublished work). In the last mentioned cases, the resistance to autolysis may be a result of the presence of additional sugars in the CW and/or the lower activity of enzymes decomposing the outer CW-layer.

RESULTS AND DISCUSSION

The total content of neutral sugars released from algal CW on hydrolysis with 2 M TFA showed considerable differences for strains of *Chlorococcales* tested in this study (Table 1). Relatively low contents were found for the CW of all the strains containing ARB.

The CWH of the strains of *Chlorella fusca* containing ARB show a 1.3 to 3.5 times higher content of total neutral sugars than the homologous CWM of the same strain. This indicates that the greater part of the polysaccharides undergoing hydrolysis are bound to the inner CW-layer.

Out of the algal strains which contain either ARB or TLS, three of them are mutants lacking ARB and ketocarotenoids. These mutant strains (C.1.1.10.14, C.1.1.10.6 and C.1.10.31) are derived from the wild strain *C. fusca* C.1.1.10 which forms ARB, TLS and ketocarotenoids. They are characterized by a high content of neutral sugars 30–47% (expressed as dry wt of CWH), and this seems to indicate that induction of a genetic defect in the formation of ARB causes an elevation of the level of polysaccharides hydrolysable by TFA. A comparable high content of total neutral sugar characterize the CW of the natural strains *C. sorokiniana* Shihira et Krauss 211–8k and *C. saccharophila* 211–1a both of which do not produce ARB. Relatively small amounts of neutral sugars are found in the three stains of *C. pyrenoidosa* (16–21%). The lowest (11–12%) levels in the CWH are, however, found in the two strains of *C. vulgaris*.

The sugar composition of CW-polysaccharides hydrolysable by 2 M TFA show large differences. Natural strains of *C. fusca* forming ARB, TLS and ketocarotenoids give rise to a CWH-neutral sugar fraction which is largely made up of mannose and glucose accompanied by some fucose. This is also true of all strains of the genus *Scenedesmus* tested and also the UV-induced mutants 6D and PG1 with defects in the carotenoid pathway, i.e. unable to form cyclic carotenoids in the absence of light but still able to make them in the light. The cell walls of these strains all contain ARB. As an additional sugar present in the CW-fraction of most *Chlorella* and *Scenedesmus* strains was component X_2. The occurence of *O*-methylxylose in CW of the *Chlorella* strains tested does not indicate any relationship with the presence of ARB.

The sugar pattern of CWH-samples derived from most natural *Chlorella* strains forming neither ARB nor ketocarotenoids show a predominance of galactose and rhamnose. The majority (six out of eight tested natural strains) not containing ARB give this sugar pattern. In one case (*Chlorella sorokiniana* 211–8k), the galactose and rhamnose content was high but sugar X_4 (unidentified) dominated. For *Chlorella* strain 137 the major sugars were rhamnose and glucose.

Chlorella mutants C.1.1.10.6, C.1.1.10.31 and C.1.1.10.14, defective in ARB, TLS and ketocarotenoids, also represent the rhamnogalactose sugar type. The detailed biochemical characteristics of these mutants were given in previous papers [3, 28]. All of the mutants contain in their CW components X_3, X_6 and X_7, compounds which are absent in the paternal strain, or present in very low nondetectable quantities. The CW of mutant C.1.1.10.14 also contained low amounts of unidentified sugars X_8 and X_9. The CW-sugar pattern of *Chlorella* mutants shows that the metabolic defects of these algae contribute to the occurrence of unusual sugars in the CW-polysaccharides. The absence of fucose in the CW is a characteristic feature of these mutants.

The CWM sample of algae containing ARB consist almost exclusively of the trilaminar structure. The ARB content of these samples is some 13–43% for *Chlorella* and 33–44% for *Scenedesmus* strains. This is the highest ARB content of all hitherto described biological structures. The existence of ARB exclusively in the TLS raises the question as to the chemical nature of the other components accompanying the ARB. The results presented in Table 1 indicate that CWM is characterized by a high content of mannose and fucose. The content of fucose in each of the CWM samples tested is higher than that of the homologous CWH sample. Additionally, the CWM of *Chlorella* contains some 3.5 times more rhamnose than the homologous CWH. Strains belonging to the genus *Scenedesmus* do not show clear differences in rhamnose content for both CW-types. The majority of *Scenedesmus* CWM (three out of four) samples are characterized by having a low mannose content and a higher galactose content. A similar tendency was found for the CW of *Chlorella* strains containing ARB. The higher content of galactose in CWM of *Scenedesmus* (when compared with CWM of *Chlorella* containing ARB) may indicate genus specific differences in the galactose content of galactose-containing polysaccharides or glycoproteins. The cell walls of the algae forming ARB do not contain sugars X_3–X_9.

The CWH of *Chlorella fusca*, mutant 1.1.10.16 and *Chlorella pyrenoidosa* strain 4 contain a very small quantity of an unidentified sugar which on GC is less mobile (7.5) than fucose (8.0). Fucose was not detected in any of

Table 1. Total content and composition of cell wall neutral sugars of chlorococcalean algae

Strains	CW type	ARB	RHA	Fuc	X	X_1	OMX	Ara	X_2	Xyl	X_3	X_4	X_5	Man	Gal	Glc	X_6	X_7	X_8	X_9	Total sugars	Collection
Chlorella fusca																						
211-8b	H	+	1.6	1.3	0	0	0.4	0.2	0.6	0.4	0	0	0	68.7	0.1	26.7	0	0	0	0	21.1	G
	M	+	9.8	21.4	0	0	3.1	1.5	0.4	3.7	0	0	0	46.5	6.8	6.8	0	0	0	0	15.7	
211-11n	H	+	4.3	2.2	0	0.4	0	2.3	0.4	1.5	0	0	0	75.0	0.1	13.8	0	0	0	0	25.1	G
	M	+	15.5	13.2	0	0.2	0	2.6	0.3	3.4	0	0	0	55.5	4.2	5.1	0	0	0	0	7.2	
211-15	H	+	4.4	4.3	0	0.6	0	1.4	0.2	2.1	0	0	0	62.4	3.7	20.9	0	0	0	0	12.3	G
	M	+	15.4	21.1	0	0.3	0.7	0.3	0.2	1.5	0	0	0	54.1	2.6	3.8	0	0	0	0	9.5	
1.1.10	H	+	5.0	2.9	0	0.5	0.2	1.1	0.1	1.4	0	0	0	70.0	3.9	14.9	0	0	0	0	16.3	Cz
	M	+	14.0	23.0	0	0.2	0.2	1.0	0.1	1.2	0	0	0	50.3	6.0	4.0	0	0	0	0	7.5	
1.1.10.6	H	0	29.5	0	0.1	2.6	5.9	2.6	0.2	5.9	7.2	0	0	4.1	34.2	6.8	0.1	0.7	0	0	47.0	Cz
	M	0	58.2	0	0	2.5	4.5	1.7	0.3	7.3	1.8	0	0	2.1	15.2	4.3	0.3	1.8	0	0	54.4	
1.1.10.31	H	0	47.6	0	0	6.3	6.8	2.6	0	7.9	1.3	0	0	3.3	19.3	3.4	0.3	1.2	0	0	50.1	Cz
1.1.10.14	H	0	52.7	0	0	0	13.2	7.8	0	5.0	3.5	0	0	3.4	6.9	2.8	0.3	3.3	0.9	0.2	30.3	Cz
C. pyrenoidosa 4																						
	H	0	25.9	0	0.1	0	0	12.2	0	7.3	1.6	0	0	14.0	38.8	0.1	0	0	0	0	16.5	SP
A24	H	0	40.0	0	0	0	8.3	12.5	0	5.5	3.6	0	0	5.7	21.2	3.2	0	0	0	0	20.2	P
137	H	0	45.8	0	0	0	0	11.7	0	4.4	0	0	0	9.9	7.1	21.1	0	0	0	0	21.5	SP
C. saccharophila																						
211-1a	H	0	29.6	0	0	0.2	1.2	2.9	0.2	3.1	0.3	0	0	22.1	39.0	1.3	0	0	0	0	47.9	G
sp.140	H	0	35.5		0	0	6.0	9.2	0	4.1	0	0	0	8.9	36.0	0.2	0	0	0	0	12.0	SP
C. sorokiniana																						
211-8k	H	0	18.9	0	0	0	0	1.4	0	10.8	0	29.6	4.1	6.8	28.4	0	0	0	0	0	47.5	G
C. vulgaris																						
211-1e	H	0	48.9	0	0	0	0	12.0	0.1	4.4	0.2	0	0	9.5	18.5	6.4	0	0	0	0	12.4	G
1890	H	0	27.2	0	0	0	0	3.4	0	14.8	0	0	0	13.3	37.7	3.7	0	0	0	0	11.5	K
Scenedesmus obliquus																						
633	H	+	8.0	9.1	0	0.3	2.3	4.1	0.2	4.6	0	0	0	52.6	5.8	13.0	0	0	0	0	10.9	B
	M	+	8.1	21.1	0	0.1	11.1	3.0	0.1	4.2	0	0	0	23.1	13.0	16.2	0	0	0	0	7.9	
6D	H	+	5.8	10.4	0	0.2	1.1	1.3	0.5	0.8	0	0	0	44.5	6.9	28.5	0	0	0	0	12.6	M
	M	+	3.5	28.3	0	0.1	1.7	0.8	0.1	0.3	0	0	0	36.8	28.3	0.1	0	0	0	0	5.8	
PG-1	H	+	3.0	7.1	0	0.2	0.3	0.8	0.2	0	0	0	0	52.2	3.7	32.4	0	0	0	0	16.0	Li
	M	+	4.8	15.7	0	0.1	0.8	0.2	0.1	0	0	0	0	56.1	20.9	1.3	0	0	0	0	12.7	
S. quadricauda																						
449	H	+	10.8	26.4	0	0	1.7	0.1	0.4	0.5	0	0	0	46.4	0.5	13.2	0	0	0	0	25.2	B
	M	+	9.8	41.2	0	0	2.1	0.1	0.1	8.5	0	0	0	22.4	13.5	2.3	0	0	0	0	7.6	

The sugar composition is given as a percentage of total sugars in the hydrolysate. The content of total sugars in percentages of dry weight of CW. Sugars are listed according to their positioning on chromatogram; H = CWH; M = CWM; ARB + = presence of ARB in CW; ARB = absence of ARB in CW. OMX = *O*-methylxylose, X–X_9 (unidentified sugars).
Data are average values of two to three analyses.

the strains defective in ARB. The results presented in Table 1 indicate a relationship between the occurrence of fucose and ARB in CW of Chlorococcales.

The quantitative differences in the neutral sugar pattern of CWH and CWM containing ARB indicate that the process of biological decomposition of CWH is accompanied by a relative loss of mannose (except for PG 1) and glucose (except for *B. obliquus*). β-1, 4-Mannanase activity has been found in CWH and homogenates of *Chlorella fusca* 211–8b [26].

Loos and Meindl [26, 29] and Walter [27] reported that an autolytic fucosidase activity also participates in the liberation of the sporangium wall, i.e. the CWM. Klinkhard [30] presented evidence to show, that this liberation is the result of the coaction of β-D-fucosidase β-D-mannosidase and β-D-fuco-β-D-glucosidase (an unique enzyme with two specificities). The combined action of these enzymes leads to the dissolution of the inner CW-layer of the initial CW composed of cellulose-like polysaccharides.

The higher content of fucose in CWM corresponding to TLS could mean that this structure contains the major part of the fucose present in the CW. However, it can not be excluded that the fucose is bound to other CW-components. Fucosidase which is activated during autospore liberation would be able to hydrolyse only a specific type of bond. There exists the possibility that a number of fucose molecules are deeply inserted in the hydrophobic macromolecule of ARB and for this reason are not accessible to the action of fucosidase. A number of fucose molecules may play the role of linkage elements between TLS and the inner CW-layer containing cellulose-like polysaccharides built from glucose and mannose units [31–33].

The characteristic sugar pattern of algal CW belonging to the *Chlorococcales* can serve as an additional criterion for the biochemical classification of the various strains.

The study of the differences in sugar composition of the cell walls obtained from various strains has been restricted to the neutral sugar fraction. This is mainly formed from hemicelluloses and glycoproteins. The quantitative differences in the neutral sugar patterns, especially for rhamnose, fucose, mannose and glucose, of the homologous CWH and CWM fractions of the various strains examined in this study seem to indicate the existence of specific ARB binding components (probably glycoproteins). Some exceptions to this trend, e.g. in the case of the mannose for the *Scenedesmus* mutant PG 1, may be caused by the presence of quantitatively significant amounts of polysaccharides or glycoproteins not related to ARB and which overlap with the ARB neutral sugar pattern. Although the results presented herein clearly show the existence of a relationship between ARB and fucose-rich CW-polysaccharides or glycoproteins which are assumed to be related with ARB deposition, one can not exclude the possibility of some exceptions. One such example seems to be the endosymbiontic *Chlorella* strain PBi susceptible to virus-infection [34]. The finding that there exists a *Chlorella PBi* virus-sensitive strain containing 6.6% of fucose in the CW with homogeneous outer CW-layer [35] supports the hypothesis that the resistance to virus depends upon the presence of TLS as well as TLS-bound fucose components and ARB. The other two sensitive *Chlorella* strains, NC 64 A and 211–81, do not contain fucose and have an homogeneous outer CW-layer like *C. vulgaris*. The simultaneous absence of ARB and the presence of fucose-rich biopolymers in the outer CW layer may also be interpreted as an example of an ARB biosynthetic defect in such strains. The fact that only a small part of the CW-glycoprotein fraction is soluble complicates investigations [31 and authors' unpublished data]. These components are of interest since the finding of Shinpo and Kunihiro [36] that algal glycoproteins obtained from *Chlorella* and *Spirulina* cells show antileukaemic activity.

EXPERIMENTAL

Algal strains. The algal strains used in this paper were obtained from the following collections: Sammlung von Algenkulturen, Universität Göttingen, F.R.G. (G): *Chlorella* strains: 211–8b, 211–11n, 211–15, 211–1a, 211–1e, 211–8k; Prof. F.-C. Czygan, Universität Würzburg, Department of Pharmaceutical Biology, (Cz): *Chlorella* wild strain C.1.1.10 forming chlorophylls, carotenoids, ketocarotenoids and ARB as well as its UV-mutants originally isolated and numbered by Allen [see 37] i.e. *mutant strains* C.1.1.10.6 (green mutant-defective in ketocarotenoids and ARB), C.1.1.10.31 (yellow mutant, not forming chlorophylls, ketocarotenoids and ARB), C.1.1.10.14 (white mutant forming neither pigments nor ARB). The detailed characterization of these mutants is given in papers [3, 28]; University of St. Petersburg-(SP): *Chlorella strain* 4, 137 and 140; Collection of Czech Academy of Sciences, Praha-(P) Chlorella strain A 24 Pädagogische Hochschule, Köthen, F.R.G.-(K): *Chlorella* strain 1890; Author's collection-(B): *Scenedesmus strains* 449 and 633; University of Marburg, F.R.G. - (M) [38]: *Scenedesmus* mutant strain 6D; University of Liverpool, U.K. - (Li): *Scenedesmus* mutant PG-1.

All strains were cultivated on medium I of ref. [40] enriched with 2.5 g each of glucose and sucrose. Growth conditions were the same as previously described [6]. For isolation of maternal cell walls (CWM), as well as for complete cell walls from homogenates (CWH) of mechanically disintegrated cells, 30-day-old cultures were used. The procedures of isolation and purification were the same as previously described [41].

Methods. Analyses of neutral sugars (as their respective alditol acetates) obtained from CW-hydrolysates prepared with 2 N trifluoroacetic acid (TFA) according to ref. [42] were carried out by FID-GC column 1.8 m × 2 mm with 3% SP-2340, N_2 as carrier gas (28 ml min^{-1}) using a temp. program of 190–225° at 2° min^{-1} [22]. Inositol (50 μl of 0.2 M soln) was used as an int. standard. The assay of the acetolysis-resistant biopolymer previously called sporopollenin was carried out on isolated cell walls by the method described previously [5].

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