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Comparison of nitrogen content amino acid composition and glucosamine content of cell walls of various chlorococcalean algae

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Abstract

Two types of ultrastructure of the outer cell wall layer of chlorococcalean algae are distinguished. The first containing the acetolysis resistant biopolymer also termed algaenan composed of polymethylenic chains. This type shows a membrane-like trilaminar structure. The second type shows homogeneous structure of the outer cell wall layer and does not contain Biopolymer.

Differences in the nitrogen-containing cell wall-components of various strains forming and not forming biopolymer (i.e. total nitrogen, amino acid pattern, as well as hexosamine content) and their relationship with the occurrence of biopolymer are presented. The paper also shows differences in amino acid pattern and glucosamine content between cell walls isolated from homogenates and cell walls of mother cells.

Keywords: Chlorococcales; Green algae; Cell wall (CW) components; N-containing compounds; Acetolysis-resistant biopolymer; Amino acid pattern; Glucosamine

1. Introduction

In recent years green algae, especially those belonging to Chlorococcales have been intensively studied as a new source of proteins for food industry and biotechnology. A strong barrier on the way of the utilization of their components is the high resistance of cell walls in some species (Burczyk, 1973). It is a structure resistant to treatment with a number of lytic enzymes e.g. cellulases, hemicellulase, pectinase, lysozyme, pronase and other enzymes as well as chemical agents (Atkinson, Gunning, & John, 1972; Burczyk, 1973). This resistance to the above treatments is caused by the presence, in the outer cell wall layer of the trilaminar structure (TLS) which is formed of several resistant components i.e. glycoproteins (Burczyk, 1973), glucosamine-containing biopolymers (Burczyk et al. unpublished data) and acetolysis resistant biopolymers (ARB) (Burczyk, 1987a) termed algeanans (Derenne, Largeau, & Hatcher, 1992; Derenne et al., 1992). Algeanans of Chlorococcales are nonhydrolyzable biopolymers resistant to acetolysis and alkaline hydrolysis. These algeanans comprise large amounts of up to C30 polymethylenic chains. They show a complete lack of isoprenoid moiety and can no longer be considered as derived from carotenoids as previously supposed (Atkinson et al., 1972; Burczyk, 1987a; Burczyk, 1987b; Burczyk & Dworzański, 1988). Such an object confirming this view is the apochlorotic alga *Prototheca wickerhami*, which contains ARB and is not able to form carotenoids at all (Puel, Largeau, & Giraund, 1987). This alga is sometimes considered as an apochlorotic form of *Chlorella* (Atkinson et al., 1972), although this view has been questioned (Lewin, 1974).

Some green algae e.g. strains belonging to the genus *Botryococcus braunii* show wider biosynthetic capabili-

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ties in respect to CW-biopolymers (Berkaloff et al., 1983). Various *Botryococcus* races A, B and L have been distinguished. They produce structurally different CW biopolymers PRB-A and PRB-B (unbranched hydrocarbons) Templier, Largeau, Casadevall, & Berkaloff, 1992) as well as PRB-L composed of saturated isoprenoid C40 unit linked by ether bridges (Metzger & Casadevall, 1987; Derenne, Largeau, Casadevall, & Berkaloff, 1989; Derenne, Largeau, Casadevall, & Sellier, 1990).

The growth of chlorococcalean algae forming ARB is accompanied by the accumulation of sporangal walls so-called cell walls of mother cells (CWM) in the medium. This results from division, size increase of daughter cells (autospores) inside the maternal cells (sporangium), and autolysis of the inner 'cellulosic layer' of complete cell walls (CW), also known as 'rigid wall' (Burczyk, Grzybek, Banaś, & Banaś, 1970; Takeda & Hirokawa, 1979a).

Cell walls of all strains belonging to the genus *Chlorella* and *Scenedesmus* forming ARB are characterized by a trilaminar structure forming the outer cell wall-layer. The ultrastructure of CWM is identical to the trilaminar structure of a complete cell wall (Burczyk et al., 1970). The CWM of the above mentioned algae contain ARB and secondary carotenoids, mainly ketocarotenoids which give the cell walls a pink coloration (Burczyk, 1970; Derenne et al., 1990). An exception is the alga *P. wickerhamii*, which does not form carotenoids at all. The function of secondary carotenoids, present in cell walls of various chlorococcalean algae, remains unexplained.

An interesting aspect of cell wall studies is to investigate the nitrogen content of both kind of cell wall preparations i.e. CWM and CWH. The comparison of N-content in the mentioned cell wall preparations indicates the location of N-containing cell wall-biopolymers accompanying the ARB inside the cell wall.

Clean preparations of CWM and CWH make it possible to study the differences in chemical composition of both cell wall types. The high ARB content in CWM (in the range of 33–42%, depending on the strain and the low content in cell walls from the homogenates of cells — CWH) makes it possible to define the chemical nature of biopolymers which accompany ARB (Burczyk, 1987a). This seems possible because components forming the inner 'cellulosic' layer have been dissolved by enzymes during autospore liberation. The remaining clean preparation of trilaminar structure i.e. the CWM consist of ARB bound to other accompanying biopolymers (probably glycoproteins and polysaccharides). These may be involved in ARB binding and deposition. It seems that their isolation and investigation will improve understanding of the mechanism of ARB deposition.

Reports about the amino acid composition of chlorococcalean cell walls are presented by (Nothcote, Goulding, & Horne, 1958; Burczyk, 1973; Takeda & Hirokawa, 1984; Takeda & Hirokawa, 1985; Takeda, 1988). Previous studies showed, that cell walls of *Scenedesmus obliquus* contain glycoproteins (Burczyk, 1970, 1973, 1987a). All these papers give evidence that cell wall-proteins of this alga — *S. obliquus* are composed of protein amino acids. In some strains of *S. obliquus* additionally hexosamine was found (Burczyk, 1973), in *Chlorella pyrenoidosa* glucosamine (Northcote et al., 1958) and also glucosamine in an alkali-insoluble cell wall fraction in one of four tested strains of *C. ellipsoidea* (Takeda & Hirokawa, 1984). Some *Chlorella* strains contain uncommon amino acids: α-HyL, 1-Me-His, β-alanine and one unidentified ninhydrin positive component was also reported (Kapaun, Loos, & Reisser, 1992).

The aim of this paper was to determine the amino acid pattern of cell wall proteins as well as to confirm the presence of other N-containing constituents forming the cell walls. It was also to study the amino acid composition of trilaminar structure of cell walls i.e. the CWM in algae containing ARB.

2. Results and discussion

The nitrogen-content in cell wall preparations was obtained by elemental analysis. This method was especially useful for CWM because of the small quantities available. The total N-content in cell wall preparations in the tested chlorococcalean strains is presented in Table 1.

These data show a remarkable differentiation of the total N-content in cell walls for various strains. This concerns both kinds of cell walls: the CWH and CWM. The highest total N-content was found in CWH of *Chlorella vulgaris* Beijerink strain 136 (7.78% N), the lowest in *Chlorella saccharophila* 211-1a (0.2% N). The ARB-containing CWH of *Chlorella* show rather a low or medium level in the range between 0.97% N for *Chlorella fusca* strain 211-11n and 2.26% N for *C. fusca* strain 211-8b.

The content of total nitrogen in CWM of ARB-forming strains of *Chlorella* is 1.5–3.9 times higher than for CWH isolated from the same strain. The results given in Table 1 indicate that the N-containing cell wall biopolymers occur mainly in the trilaminar structure which is located in the outer part of the cell wall. Cell walls isolated from strains which do not contain TLS and ARB also show a higher content of N-containing biopolymers in the outer cell wall layer when compared with CWH of the same strains. Such examples include: the *Chlorella* mutant 1.1.10.6 which was isolated by (Bendix & Allen, 1962) and is characterized by the N_{CWM}/N_{CWH} ratio 1.32, as compared to

Table 1
Elementary composition and glucosamine content (%) of cell walls of various algal strains belonging to *Chlorococcales* forming — and not forming - ARB. CWH — cell walls isolated from homogenate; CWM — cell walls of mother cells accumulated in the medium; ARB+ = presence of ARB in CW; ARB (0) is absence in CW; n. — not determined; N_1/N_2 — ratio of total nitrogen content (%) in CWM/CWH * — strain not accumulating CWM in the medium; (a) — difference to 100% hold of; ash, phosphorus and evtl. sulfur content

Strains	Cell-wall type	ARB	N	O	C	H	S	Ash	N_1/N_2	Glucosamine (% of CW d.w.)	Collection
Chlorella fusca 211-8b	CW M	+	3.35	18.29	60.65	9.30		8.41	1.48	2.85	G
	CW H	+	2.26	29.96	50.33	7.53		9.92		2.93	
211-8p	CW M	+	3.42	18.67	63.59	9.75	1.44	3.13	1.91	2.71	G
	CW H	+	1.79	31.60	49.28	7.48	0.78	9.07		2.81	
211-11n	CW M	+	2.63	19.73	59.47	9.30		8.87	2.71	2.79	G
	CW H	+	0.97	34.20	46.72	7.12		10.99		2.30	
211-15	CW M	+	3.76	20.40	57.81	8.84		9.19	2.54	3.28	G
	CW H	+	1.48	34.20	47.87	7.20		9.25		2.95	
113	CW M	+	4.73	18.83	60.43	9.17		6.84	3.9	4.17	SP
	CW H	+	1.20	34.67	46.84	7.00		10.29		2.77	
620	CW M	+	4.46	19.42	64.92	8.87	1.21	2.18	3.28	n	B
	CW H	+	1.36	38.36	44.59	6.52	0.43	8.25			
C.1.1.10	CW M	+	4.66	18.67	61.04	9.39		6.24	3.11	n.	Cz
	CW H	+	1.50	37.70	45.79	6.89		8.12		0.67	
C.1.1.10.6 (mutant)	CW M	0	1.77	n.	41.89	5.86		n.	1.32	n.	Cz
	CW H	0	1.34	n.	42.53	6.01		n.		4.88	
C.1.1.10.31 (mutant)	CW H	0	2.67	n.	42.55	6.26		n.	*	3.94	Cz
C.1.1.10.14 (mutant)	CW H	0	2.01	39.66	41.17	6.24		10.92	*	2.59	Cz
Chlorella pyrenoidosa A-24	CW H	0	5.26	n.	39.74	5.58		n.	*	3.55	P
Chlorella saccharophila 211-1a	CW H	0	0.20	39.46	39.18	5.97		15.19	*	2.80	G
211-9a	CW H	0	2.16	41.06	40.30	6.04	3.88	7.56	*	3.88	G
8	CW H	0	2.23	39.11	42.21	6.42	1.33	8.7	*	3.91	SP
Chlorella sorokiniana 211-8k	CW H	0	5.66	34.53	39.25	5.87		14.60	*	18.48	G
137	CW M	0	5.25	36.30	43.66	5.92	2.18	6.69	1.47	17.38	SP
	CW H	0	3.56	35.58	42.50	5.45		12.71		16.20	
Chlorella vulgaris Gromov 140	CW H	0	4.72	35.28	34.63	5.63	1.99	17.75	*	21.28	SP
Chlorella vulgaris Beijerinck B	CW H	0	3.03	39.20	35.11	5.27		17.39	*	16.58	SP
136	CW H	0	7.78	32.55	38.40	5.80	2.15	14.34	*	17.64	SP
153	CW H	0	5.49	38.27	35.85	5.68	2.06	14.86	*	17.16	SP
211-1e	CW H	0	4.10	37.02	34.71	5.96	4.69	14.59	*	22.33	G
Scenedesmus obliquus 633	CW M	+	5.49	18.90	55.67	8.20	0.96	11.15	1.58	3.02	B
	CW H	+	3.58	31.21	48.08	7.10	0.90	9.79		2.09	
6D (mutant)	CW M	+	6.21	19.87	55.15	8.25		10.52	1.74	2.79	M
	CW H	+	3.55	27.50	47.92	7.13	1.12	13.99		1.67	
PG 1 (mutant)	CW M	+	7.03	18.85	56.96	8.43	1.45	8.16	2.14	2.37	Li
	CW H	+	3.29	29.21	47.88	7.18	0.96	11.99		1.46	

the original wild strain *C. fusca*, strain 1.1.10 for which the ratio N_{CWM}/N_{CWH} was 3.11.

Natural strains that do not contain ARB and accumulate CWM are relatively rare. *C. pyrenoidosa*, strain 137 is one of such rare examples. It is characterized by the low value of N_{CWM}/N_{CWH} ratio 1.47. This strain accumulates small quantities of CWM in culture medium. It seems that the other natural strains tested, which do not form ARB, dissolve their cell walls by autolytic enzymes after liberation in the medium. The high ash content in some cell wall samples may be a consequence of strain-specific chemical absorption of cations to CW-biopolymers. This absorption seems to be, in most cases, higher for CWH-samples.

The comparison of amino acid pattern of CWH and CWM of the same *Chlorella* and *Scenedesmus* strains (Figs. 1 and 2) forming ARB shows similarities. Both kinds of cell wall preparations contain amino acids typical for proteins. This confirms previous findings (Northcote et al., 1958; Burczyk, 1973; Takeda & Hirokawa, 1984; Takeda & Hirokawa, 1985) that proteins, but not peptidoglycans, as in the case of blue–green algae, participate in the formation of the cell walls of chlorococcalean algae. Tryptophan and sulfur-containing amino acids i.e. cysteine and methionine were not taken into account because they were generally destroyed in the course of acid hydrolysis with 6 N HCl, applied in this study. The quantitatively prevailing amino acids in CWH of *Chlorella* strains forming ARB are the following: aspartic acid, glutamic acid, leucine and alanine. In CWM from the same strains prevail: aspartic acid, alanine, threonine, serine

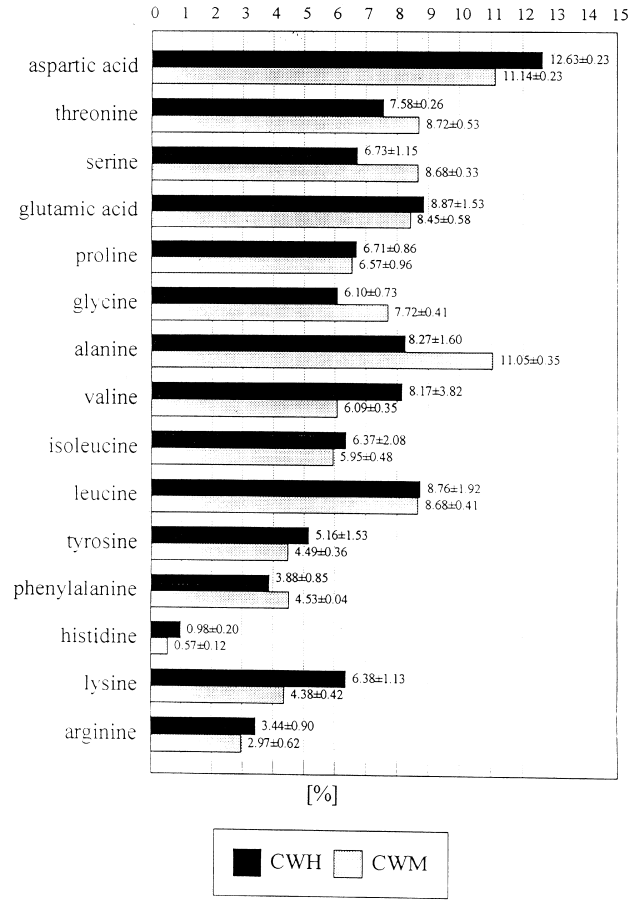

Fig. 1. Average of two to three analyses of CW-samples isolated from *Chlorella fusca* strains: 211-8b, 211-8p, 211-11n, 211-15, 113 and C.1.1.10.

and leucine. The CWM of the above mentioned strains show 1.15-fold higher content of threonine, 1.29× serine, 1.27× of glycine, 1.34× of alanine and 1.17× of phenylalanine (see Fig. 1).

The content of basic amino acids in *Chlorella* CWM as compared with the CWH of the same strains is lower for: histidine 1.72 × , lysine 1.46 × , arginine 1.16× (when calculated CWM/CWH content). The level of remaining amino acids is comparable for both kinds of cell walls. *Scenedesmus* CWH shows higher content of all basic amino acids than CWM of the same strain (see Fig. 2).

The comparison of the percentage of amino acids in CWH of ARB-forming and -not forming *Chlorella* strains is presented in Fig. 3. These data are average values of amino acid analyses of cell walls derived from six *Chlorella* strains containing ARB and 11 *Chlorella* strains not forming ARB. The largest differences in amino acid pattern concern the following basic amino acids: lysine, arginine and histidine whose content is respectively, 2.19 × , 2.16× and 2.26× higher as compared with CWH of ARB-containing strains. The content of phenylalanine is 1.27 times higher for strains without ARB. The opposite trend is shown by all the remaining amino acids except tyrosine, it is most pronounced in the case of alanine, aspartic acid and threonine.

Differences in amino acid content of CWM and CWH concern strains forming ARB (Fig. 2). In our opinion, they are the effect of autolytic changes occurring during autospore liberation. In the course of analysis, some amino acids may be split of by the proteases from the peptide chains forming the cell wall structure.

The amino acids pattern of the several strains investigated here confirms the previous results (Northcote et al., 1958; Takeda & Hirokawa, 1979bTakeda & Hirokawa, 1984; Takeda, 1988) that cell walls of chlorococcalean algae are formed of 15 amino acids in contrast to cell walls of blue–green algae (Salton, 1960) containing the muropeptide which is composed of 4–5 amino acids. This reflect the higher organization level of cell wall of chlorococcalean algae when compared with blue–green algae.

The differences of average content of amino acids in cell wall samples derived from *Chlorella* and

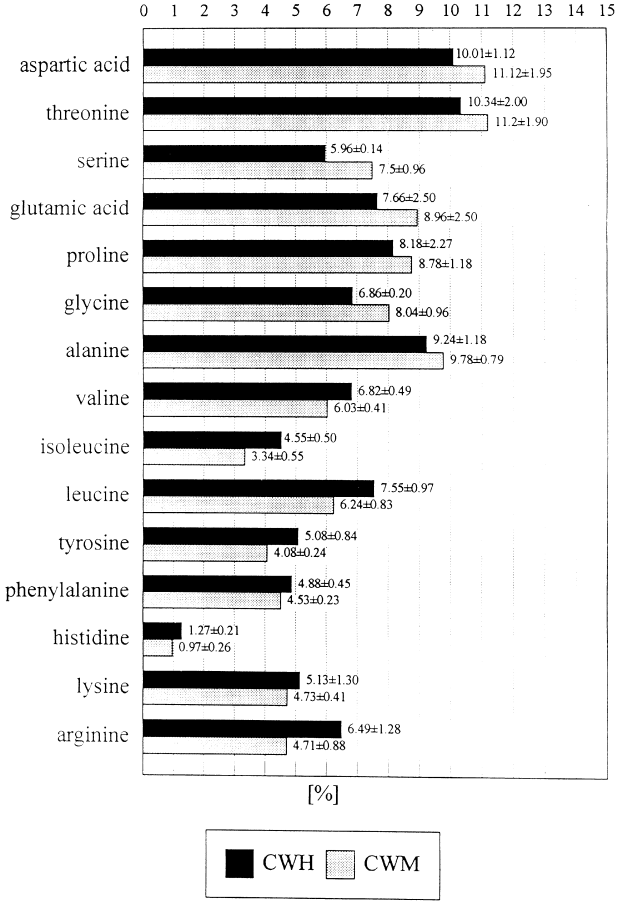

Fig. 2. Average of three analyses of CW-samples isolated from *Scenedesmus obliquus*, strain: 633, 6D, PG 1.

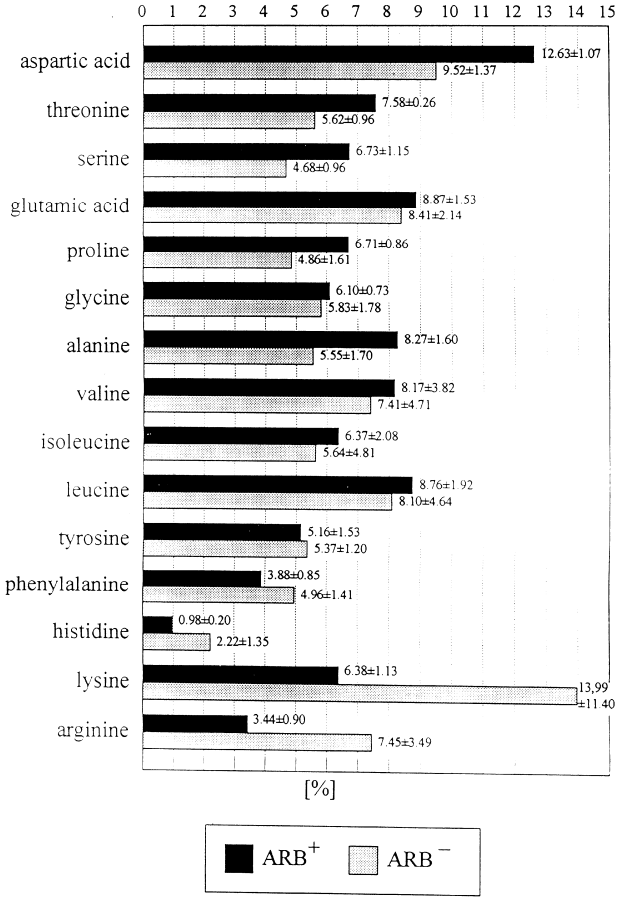

Fig. 3. Average of two to three analyses of CWH-samples isolated from *Chlorella* strains forming ARB: 211-8b, 211-8p, 211-11n, 211-15, 113 and C.1.1.10 and isolated from strains not forming ARB i.e. 211-1a, 211-9a, 8, 211-8k, 137,140, B, 136, 153, 211-1e.

Scenedesmus, presented here, have been verified by means of computerized statistical procedures using the Cochrana-Coxa test. The significance of differences have been verified by Statistica program on the confidence level $\alpha = 0.05$.

The amino acid pattern of CWM reflects the composition of proteins in the TLS. They are assumed to be related with ARB deposition. Among CWM proteins some enzymatic activities as: phosphatases, glycosidases and esterases have been observed (Burczyk & Loos, 1995). However, the full spectrum of proteins (glyco- or glyco–lipo-proteins) related to the deposition of ARB and other enzymatic activities remaining in the CWM after their liberation in the medium remains unknown.

Our chromatograms confirmed the presence of a ninhydrin-positive component in all investigated cell wall samples Table 1. It is absent in hydrolysates of pure protein e.g. bovine albumin. The location of this component on the chromatograms between phenylalanine and histidine corresponds to that of glucosamine. Glucosamine was first detected by (Mihara, 1961) in the residue of cell walls of *C. ellipsoidea*, previously extracted with perchloric acid. The authors suggest that glucosamine is derived from cell wall chitosan. Its content remains constant during the growth phase and increases only in the reproductive phase of the *Chlorella* cell cycle i.e. during the formation of autospore cell walls. The glucosamine content makes up 86% of the total amino groups of the rigid layer of *C. ellipsoidea* (Takeda & Hirokawa, 1979b).

Our experimental data show that glucosamine found in hydrolysates prepared obtained were in 3 or 6 N HCl hydrolysis may be a degradation product of biopolymers containing of *N*-acetyl-glucosamine. Such an opinion is confirmed by the enzymatic degradation of *Chlorella* sp. strain Pbi cell walls by chitinase of *Streptomyces* sp. (Kapaun et al., 1992).

Attempts to define the location previously of *N*-acetyl-glucosamine in the cell walls of *Scenedesmus acuminatus* were carried out with a lectin reacting with *N*-acetyl-glucosamine i.e. the wheat germ agglutinin labelled with gold. These experiments showed that *N*-acetyl-glucosamine is included in Golgi vesicles and then found in oligosaccharides of the plasma membrane. The intensity of cell wall labelling was, however, relatively low (Hayashi & Ueda, 1987). This finding seems to correspond well with the low content of glucosamine in cell walls derived from the *Scenedesmus* strains investigated in this paper.

Glucosamine was found in cell walls of *C. pyrenoidosa* (Northcote et al., 1958), *C. ellipsoidea* (Takeda & Hirokawa, 1979b) as alkali-soluble fraction. The latter authors distinguished it from chitin, which is insoluble in alkali.

It seems that glucosamine may participate in the glycosylation of peptides and/or polypeptides and forms, in the cell wall, a network between these components and carbohydrate chains. Solubilization of glucosamine-containing component by aqueous solutions of sodium hydroxide, commonly used not only for protein solubilization and protein estimation (Lowry, Rosenbrough, Ferr, & Randall, 1951), but also for extraction of hemicellulose (Takeda & Hirokawa, 1978), may indicate that glucosamin is bound in cell walls with both proteins and polysaccharides and exists in glycoproteins.

A general implication of our results is that glycoproteins- and glucosamine-containing biopolymers are very common among chlorococcalean algae and are located not only in the inner cell wall layer, known as 'rigid wall' (Takeda & Hirokawa, 1978; Takeda, 1993), but also in the outer cell wall layer. This concerns both cell wall types containing and not containing ARB Table 1. Our data show that the presence of ARB in the outer cell wall layer i.e. in the trilaminar structure of chlorococcalean CW is somehow negatively correlated with low level of glucosamine-contain-

ing biopolymers. The glucosamine content of CWM of majority of strains containing ARB is higher than in CWH. This concerns also the *Chlorella* strain 137 not containing ARB. The *C. fusca* strains 211-8b and 211-8p showing a comparable content of glucosamine in both cell wall types may be considered as exceptions. In the case of *C. fusca* strains 211-8b and 211-8p the higher activity of enzymes dissolving the glucosamine-containing biopolymers may be responsive for glucosamine-content lower than in CWH of the same strain. It is well known, that the process of autospore and CWM liberation is accompanied by the enzymatic lysis of the inner layer of sporangium and probably also a partial dissolution (or cleavage) of outer layer components. This process may be strain-dependent and probably leads to decrease of the glucosamine content in CWM in comparison to the original glucosamine level in the outer cell wall layer of the living cells. In fact, it has been confirmed the accumulation of the high molecular glucosamine containing cell walls biopolymers in the growth medium of this algae showing serological cross-reaction with antibodies against *Scenedesmus* — CWH (Burczyk, 1973; Burczyk et al., unpublished data).

The glucosamine-containing biopolymers of the cell wall, especially of their outer layer may play a protective role for these algae and may contribute to their resistance. In the remaining strains, which do not contain ARB, two groups can be distinguished depending on glucosamine content. The first group is characterized by low glucosamine content. These are mutants: C.1.1.10.6; C.1.1.10.31; C.1.1.10.14, *C. pyrenoidosa* A-24, *C. saccharophila* strains 211-1a, 211-9a and strain 8. Their glucosamine content is 2.59–4.88%. The second group shows, in contrast, a high content of glucosamine in a CWH: *C. sorokiniana* 211-8k, 137 as well as *C. vulgaris*, strains 136, 140, 153 and 211-1e. They contain 16.20–22.33% of glucosamine. The *Chlorella* strain 137 previously termed *C. pyrenoidosa* (Burczyk, 1982) was reexamined by Professor E. Kessler (Germany) and defined as *C. sorokiniana*.

The chemical nature of glucosamine-containing CW-biopolymers needs further elucidation. It seems that their presence in cell walls contribute to their resistance against factors which would be able to decompose the inner cell wall layer, of whole cells. An example of strain with very low N-content in the cell wall is the *C. saccharophila* strain 211-1a (which contains 0.2% N, Table 1). The cell walls of this strain are the most susceptible to the action of cellulolytic enzymes and protoplasts formation (Göbel & Aach, 1985). This low N-content is responsible for enzymatic susceptibility of this strain. An interesting aspect of cell wall investigations would consist in the exploration of other strains with cell walls susceptible to enzymatic treatment in order to check the relation between susceptibility to enzymatic action and low N-content.

3. Experimental

3.1. Algal strains

The algal strains investigated here were obtained from: Sammlung von Algenkulturen, Universität Göttingen, Germany (G): *C. fusca* var. *vacoulata* Shihara et Krauss strains: 211-8b, 211-11n, 211-15, *C. saccharophila* 211-1a, 211-8k and *C. vulgaris* 211-1e; Professor 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 induced by Allen (see Bendix & Allen, 1962) 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), white mutant which does not form pigments nor ARB) these mutants are characterized in (Göbel & Aach, 1985; Burczyk, Termińska-Pabis, & Śmietana, 1995). University of St. Petersburg (SP): *Chlorella* strain 8, 113, 136, 137, 140, 153 and B; Collection of Czech Academy of Sciences, Praha (P); *Chlorella* strain A-24: author's collection (B): *C. fusca* strain 620, *Scenedesmus* strain 633; University of Marburg, Germany (Burczyk et al., 1995) *Scenedesmus* mutant strain 6D; University of Liverpool, UK (Li), *Scenedesmus* mutant PG-1.

The above-mentioned strains were grown on medium 1 of Kessler and Czygan (1970) enriched with 2.5 g/dcm^3 each of glucose and sucrose. The growth conditions were described before by (Burczyk, 1987b). In order to isolate maternal cell walls of mother cells (CWM) and complete cell walls isolation from homogenates (CWH) of mechanically disintegrated cells, 30-day-old cultures were harvested. The procedures of isolation and purification were described earlier (Burczyk, Szkawran, Zontek, & Czygan, 1981).

4. Methods

4.1. Elemental analysis

Lyophilised CW samples were dried over P_2O_5 in vacuum. In cases where sulfur was estimated, an automatic analyzer (Perkin-Elmer Model 2400) series 2 was used. In the other cases, analyses were carried out on Carlo Erba Model EA 1108, 0.5–1.0 mg samples were analyzed for C, N, H, O content. Theoretical accuracy of the method was 0,3%. The analyses given in Table 1 are averages of two to three estimations.

4.2. Hydrolysis of CW

Hydrolysis of CW-samples with 6 N HCl was performed at 105°C for 1 h under N_2-atmosph. Amino acids were determined in an amino acid analyzer with ninhydrin detection using an Automatic Carlo Erba Analyzer AAA-3a 30 and program for hydrolysates, i.e. sodium citrate buffer pH 2.2 with the elution profile: 0.15 M Na-citrate buffer (pH 3.42 for 5 min), 0.20 M Na-citrate buffer (pH 4.0 for 22 min) and 0.2 M Na-borate buffer 0,2 M (pH 10.0 for 20 min). The analyses were carried out at 50° for 9 min and then continued at 70°.

Glucosamine HCl (Fluka, Suisse) was used as standard for estimation.

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