Polyamines in Cell Walls of Chlorococcalean Microalgae

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Biotechnology of microalgae represents a very attractive alternative as a source of energy and substances of high value when compared with plant cultivation. Cell walls of green microalgae have an extraordinary chemical and mechanical resistance and may impede some steps in the biotechnological/industrial exploitation of algae. The aim of the present contribution was to check the presence of polyamines in the cell walls of chlorococcalean green microalgae. Polyamines are nitrogenous compounds synthesized normally in cells and may affect the properties of the cell wall. Our work included strains either forming or not forming the polymer algaenan, allowing us to conclude that algaenan is not a prerequisite for the presence of polyamines in the cell walls. Polyamines were detected in isolated cell walls of *Scenedesmus obliquus*, *Chlorella fusca*, *Chlorella saccharophila*, and *Chlorella vulgaris*. Their concentration in isolated cell walls ranged between 0.4 and 8.4 nmol/mg dry weight.

Key words: Polyamines, Cell Wall, Microalgae

Introduction

Green microalgae belonging to the genera *Chlorella* and *Scenedesmus* (Chlorococcales) are subject of increasing interest in biotechnology because they have several appealing traits. Among these are their photoautotrophy, fast growth, accumulation of large quantities of biomass and many valuable cell components. During the last decades, the interest in algal biotechnology has focused on the use of microalgae as a source of bioenergy, such as biodiesel, bioethanol, biogas, and hydrogen (Papazi *et al*., 2012).

The extraordinary resistant cell wall of algae is often an obstacle to their biotechnological/industrial exploitation. The resistance of the cell wall is due to resistant biopolymers of different chemical structures, such as algaenan, a hydrocarbonaceous biopolymer previously called sporopollenin (Burczyk and Dworzański, 1988), nitrogen-containing biopolymers composed of aminosugars, such as chitosan and similar polysaccharides (Burczyk *et al.*, 1995; Hatano *et al.*, 1992), furthermore glycoproteins (Burczyk, 1973, 1987a, b; Burczyk *et al.*, 1999), hemicelluloses, cellulose, or cellulose-like biopolymers. The above mentioned biopolymers are cross-linked by chemical bridges of various types, *i. e.* -O- (ether, ester, and glycosidic bonds), -CO-NH-, and -S-S-, and they are accompanied by lipids. Their presence significantly reduces the accessibility of the cell wall to enzymatic or chemical hydrolysis as well as to mechanical rupture.

A cell wall transglutaminase is involved in the assembly and insolubilization of cell walls (Waffenschmidt *et al*., 1999). Transglutaminases attach polyamines (PAs) to specific glutamine residues of proteins, and either increase their positive charge or cross-link different domains of the same peptide or of different peptides (Ioannidis *et al*., 2012a). PAs are low-molecular weight aliphatic amines and are known by their trivial names putrescine (Put), spermidine (Spd), and spermine (Spm), respectively. Put has two amino groups, Spd has two amino and one

imino group, and Spm has two amino and two imino groups. In green algae the cellular levels of PAs are regulated by light (Kotzabasis *et al*., 1999). Their elevation during stress in green algae confers tolerance against UVB radiation (Sfichi-Duke *et al*., 2008). In photosynthetic organisms, they are involved in several processes such as the modulation of the proton motive force in thylakoids (free forms of PAs) (Ioannidis *et al*., 2012b), the structure of PSII (Kotzabasis *et al*., 1993a), the quenching of antenna fluorescence (Ioannidis *et al*., 2011), and the formation of grana (bound forms of PAs) (Ioannidis *et al*., 2009).

The presence of PAs was confirmed in the bacterial peptidoglycan of the cell wall of *Anaerovibrio lipolytica* (Hirao *et al*., 2000) and of *Selenomonas ruminantium* (Takatsuka and Kamino, 2004). Long-chain PAs occur in the cell wall of the diatoms *Cylindrotheca* and *Nitscha*, where they control the silica morphology (Kröger *et al.*, 2000). In plants, a membrane-bound enzyme, β-1,3-glucan synthase, is strongly activated by PAs; acting synergistically with Ca^{2+}, it contributes to the formation of β-1,3-glucan-containing callose barrier structures deposited in cell walls as a response to stress (Kauss and Jeblick, 1986). Cell walls of vegetative cells and the zygote of *Chlamydomonas reinhardtii* (Volvocales, Chlorophyta) also contain PAs.

Microalgal strains belonging to the Chlorococcales are characterized by two major types of cell wall structure. The differences concern mainly the outer cell wall layer (Fig. 1, O) (Burczyk, 1982). Microalgae contain an acetolysis-resistant biopolymer, called algaenan (Fig. 1a). This layer is characterized by a trilaminar structure (TLS). Furthermore, this layer remains behind as the so-called maternal cell wall (CWM) after liberation of the spores into the medium and can be isolated by centrifugation (Burczyk, 1982). The majority of natural strains without algaenan do not accumulate the CWM in the culture medium. This is probably caused by autolytic enzymes which dissolve the cell walls completely during and/or after spore liberation. In exceptional cases they remain in the medium and could be isolated (Burczyk *et al.*, 1999). The inner cell wall layer (Fig. 1, I) is dissolved in both types of microalgae by autolytic enzymes during the process of cell division and liberation of spores (daughter cells).

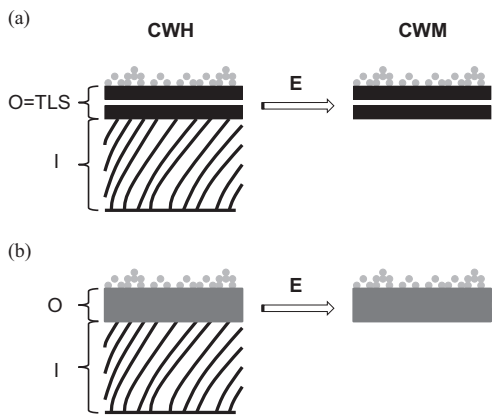

Fig. 1. Simplified scheme of CWH and CWM in chlorococcalean algae according to Burczyk (1982). The algae contain the acetolysis-resistant biopolymer algaenan in a layer with a trilaminar structure (TLS) that forms the outer layer (O) and stains densely in electron micrographs, while the inner layer (I) stains less intensely. (a) Strains containing algaenan. (b) Strains without algaenan. E, enzymes degrading the cell wall during the liberation of spores. The grey globules represent glycoproteins on the cell surface.

Previous papers by some of us (Burczyk, 1987a, b; Burczyk *et al*., 1999) have shown that the outer cell wall layer of microalgae contains 33 – 41 % algaenan, 2.63 – 7.03 % total nitrogen (glycoproteins, amino sugars), and lipids. These components contribute to the high resistance of cell walls to enzymatic lysis and extraction of cell inner components for commercial purpose. The CWM may contain some quantity of a cellulose-like biopolymer (contamination originating from the inner cell wall layer which was not completely dissolved) (Burczyk, 1987a).

In the work presented here we employed microalgal strains that either contain or do not contain algaenan in their cell walls (Burczyk and Hesse, 1981) (Table I). The algaenan-forming strains are: *Scenedesmus obliquus* strain 633 and two strains of the species *Chlorella fusca*, 211-8p and C.1.1.10 (Cz1), which accumulate CWM in the growth medium. In addition, whole cell walls (CWH) can be obtained from the homogenates of mechanically disrupted cells. The aim of the present contribution was to examine the presence of PAs in the cell walls of chlorococcalean green microalgae. To the best of our knowledge, there are no reports relating PA occurrence to the presence or absence of algaenan, a compound which significantly fortifies cell walls against chemical and enzymatic hydrolysis as well as mechanical disruption.

Materials and Methods

Organisms

The following strains were used: *Chlorella fusca*, strain 211-8p (from the algal collection of the Uni-

versity of Göttingen, Göttingen, Germany); *Chlorella fusca*, strain C.1.1.10 (Cz1) (from the collection of Prof. F.-Ch. Czygan, University of Würzburg, Department of Pharmaceutical Biology, Würzburg, Germany).

Algae devoid of algaenan were: *Chlorella vulgaris*, strains No. 136, 137, and 140 (Gromov) from the culture collection of the University of St. Petersburg, St. Petersburg, Russia. The *Scenedesmus obliquus* strain 633 was from the collection of one of the authors (J. B.).

The above mentioned strains were grown in the medium described by Burczyk (1987b), containing 2.5 g/l each of glucose and saccharose. Growth conditions were mixotrophic and aseptic (Burczyk, 1987b).

Isolation of CWM and CWH

For isolation of maternal cell walls (CWM) from culture supernatants, as well as of whole cell walls (CWH) from homogenates of mechanically disrupted cells, 30-day-old cultures were used. The procedures of isolation and purification were the same as previously described (Burczyk *et al.*, 1981).

Extraction and HPLC analysis of polyamines

Lyophilized CWH and CWM samples were extracted as previously described and analysed following the procedure of Kotzabasis *et al.* (1993b). Briefly, for PA analysis samples were suspended in 1 M NaOH (20 mg/ml) for about 2 h. An 0.5-ml aliquot of the hydrolysate was mixed with 36% HCl in a ratio of 1:1 (v/v), kept in screw cap tubes, and hydrolyzed at 110 °C for 18 h. Samples were dried at 70 – 80 °C and re-dissolved in 0.2 ml of 5% (v/v) perchloric acid. The dried products were re-dissolved in 0.2 ml of 5% (v/v) perchloric acid. For PA analysis, the samples were derivatized by benzoylation, as previously described (Kotzabasis *et al.*, 1993b). For this, 1 ml of 2 M NaOH and 10 μl benzoylchloride were added to 0.2 ml of the hydrolysate and the mixture vortexed for 30 s. After 20 min of incubation at room temperature, 2 ml of saturated NaCl solution were added to stop the reaction. The benzoylpolyamines were extracted three times into 2 – 3 ml diethyl ether, the diethyl ether phases combined, and evaporated to dryness. The benzoylpolyamines were re-dissolved in 0.2 ml of 63% (v/v) methanol, and 20-μl aliquots were analysed, as described previously (Kotzabasis *et al.*, 1993b), in a Shimadzu liquid chromatography system (LC-10AD) equipped with an SPD-M10A diode array detector (Shimadzu, Kyoto, Japan) and a narrow-bore column (C18, 2.1 mm × 200 mm, 5 μm particle size, Hypersyl; Hewlett-Packard, Palo Alto, CA, USA).

Results and Discussion

Cell walls, *i. e.* CWM and CWH, were isolated from the microalgal strains listed in Table I. From these samples the major PAs – Put, Spd, and Spm – were extracted and analysed. PAs were detected in the cell wall of all strains (Fig. 2). The highest content of total PAs was found in the *Chlorella* samples, especially in the sample CWH 140 of *C. vulgaris* (Fig. 2).

Sample 1 (CWM) of *Scenedesmus* is a preparation containing only the outer trilaminar structure of the CWH. These structures contain approx. 37% of algaenan (Burczyk, 1987a) and 5.5% of total cell wall nitrogen (Burczyk *et al.*, 1999). Because of the relative low content of glucosamine (3% of cell wall dry weight) this nitrogen content is mainly due to N-containing groups of glycoproteins. The ratio of the

Table I. CWM (samples 1, 3, 5) and CWH (samples 2, 4, and 6 – 10) from different algal strains. Strains in samples 1 – 6 contain algaenan, those in samples 7 – 10 do not.

Sample no.	Species	Algaenan	Code
1	CWM 633 (*Scenedesmus obliquus*)	+	CWM 633
2	CWH 633 (*Scenedesmus obliquus*)	+	CWH 633
3	CWM 211-8p (*Chlorella fusca*)	+	CWM 211-8p
4	CWH 211-8p (*Chlorella fusca*)	+	CWH 211-8p
5	CWM Cz1 (*Chlorella fusca*)	+	CWM Cz1
6	CWH Cz1 (*Chlorella fusca*)	+	CWH Cz1
7	CWH 140 (*Chlorella vulgaris*)	–	CWH 140
8	CWH 136 (*Chlorella vulgaris*)	–	CWH 136
9	CWH 137 (*Chlorella vulgaris*)	–	CWH 137
10	CWH 211-1a (*Chlorella saccharophila*)	–	CWH 211-1a

total nitrogen content of CWM/CWH is about 1.6 (Burczyk *et al.*, 1999). Similarly, the ratio of the total nitrogen content of CWM/CWH was 1.9 and 3.1 for strains 211-8p and Cz1, respectively, of *Chlorella fusca* (Burczyk *et al.*, 1999). The small size of the trilaminar layer (CWM, about 15 nm) in the context of the CWH (80 – 150 nm), on the one hand, and the comparable contents of PAs in CWM and CWH [expressed in nmol/mg dry weight (DW), see Fig. 2] indicates that the cell wall PAs are more or less uniformly distributed in cell walls. In other words, the presence of the additional inner layer in CWH does not increase the relative content of PAs in cell walls. Additionally, the comparative results of Fig. 2 show that the presence or absence of algaenan is not a prerequisite for the presence of PAs in the cell walls.

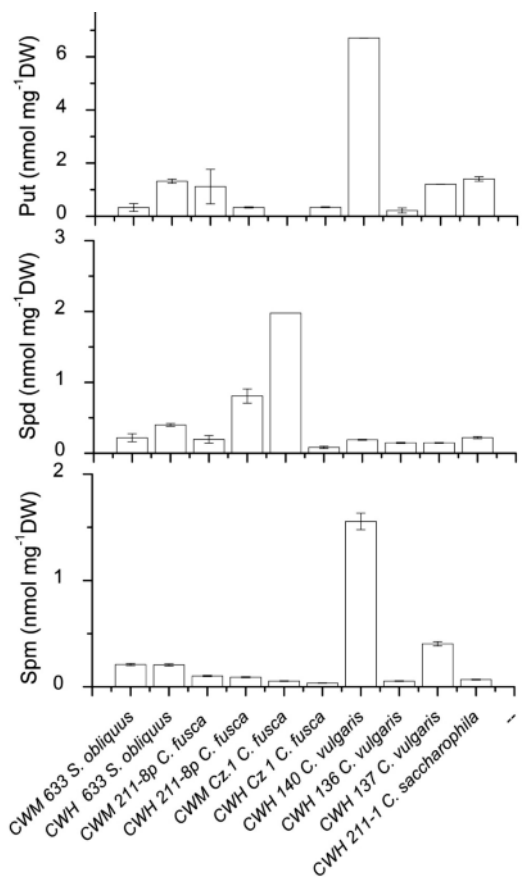

Fig. 2. Contents of putrescine (Put), spermidine (Spd), and spermine (Spm) in the cell wall samples CWM or CWH from chlorococcalean microalgae. Bars indicate SD ($n = 3$).

PAs have been found in cell walls of the green microalga *Chlamydomonas reinhardtii* (Volvocales) where they function as cross-links in vegetative and zygotic cell walls. PAs added to the growth medium of this alga act as competitive inhibitors of the cell wall protein cross-linking activity of extracellular transglutaminases (Waffenschmidt *et al.*, 1999). Several glutamine residues that are available in cell wall glycoproteins of *Chlamydomonas*, such as in GP2 (Voigt *et al.*, 2007), could serve as binding sites for PAs. A similar role of PAs might be expected in chlorococcalean algae. In higher plants, multiple negatively charged sites are available in cell wall components (Edreva, 1996), such as uronic acid residues in pectins and phenolic residues in lignins (phenolic groups are very weak acids) (Varner and Lin, 1989). Electrostatic interactions of PAs with these components mediate their binding to cell walls (Goldberg and Perdrizet, 1984; Bors *et al.,* 1989; Bagni, 1989; Slocum, 1991). More detailed information is lacking, but regulation of the cell wall rheology by PAs is strongly possible. In the majority of the investigated samples the PA content is low, which indicates that the contribution of PAs to total cell wall cross-linking and stabilization may be low. In contrast to the generally low PA content in the cell wall samples, that in *Chlorella vulgaris* strain 140 is nearly 10 times higher. In this case one can assume their contribution to cross-linking of cell wall biopolymers, similar to the mentioned cell walls of *Chlamydomonas reinhardtii* (Waffenschmidt *et al.*, 1999).

The content of PAs (especially of the tetramine Spm) in the CWH sample of *Chlorella saccharophila* 211-1a (Table I, no. 10) is very low with ca. 0.08 nmol Spm/mg DW (Fig. 2). It is the strain used by Aach *et al.* (1978) for the production of protoplasts by an enzyme mixture. This suggests, that the low level of PAs might allow better action of lytic enzymes. It seems that a better knowledge of the chemical composition of the microalga cell wall will help to elaborate more effective methods for their lysis.

Concluding remarks

PAs (Put, Spd, and Spm) were detected in isolated cells walls, *i. e.* CWM and CWH, of *Scenedesmus obliquus*, *Chlorella fusca*, *Chlorella saccharophila*, and *Chlorella vulgaris* in the concentration range of 0.4 – 8.4 nmol/mg DW. The presence or absence of algaenan is not a prerequisite for the presence of PAs in the cell walls.

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