Arch Microbiol (1982) 132:10–13

Archives of
Microbiology

Comparative Studies on *Chlorella* Cell Walls: Induction of Protoplast Formation

Takashi Yamada and Kenji Sakaguchi

Mitsubishi-Kasei Institute of Life Sciences, 11 Minamiooya, Machida-shi, Tokyo 194, Japan

Abstract. Among 12 strains of *Chlorella ellipsoidea*, *C. vulgaris*, and *C. saccharophila* tested, 4 strains (1, *C. ellipsoidea*; 2, *C. vulgaris*; 1, *C. saccharophila*) formed osmotically labile protoplasts after treatment with mixtures of polysaccharide degrading enzymes. The relationship between enzymatical digestibility and structure or composition of *Chlorella* cell walls were studied by electron microscopy and staining techniques with some specific dyes. The cell wall structures of the 12 *Chlorella* strains were grouped into three types: (1) with a trilaminar outer layer, (2) with a thin outer monolayer, and (3) without an outer layer. Protoplasts were formed only from the strains with a cell wall of Type 2. In the strains with a cell wall of Type 1, the outer layer protected the inner major microfibrillar layer against enzymatic digestion. The cell wall of Type 3 was totally resistant to the enzymes; the chemical composition of the cell wall would be somewhat different from that of other types.

Key words: Calcofluor White – Cell wall structure – *Chlorella* – Electron microscopy – Protoplast – Ruthenium Red

Over a long time, strains of the green alga *Chlorella (Chlorococcales)* have served as model organisms in plant physiological and biochemical studies because of their simple structure, simple life cycle, rapid growth under various conditions, and modest nutritional requirements. However, studies on *Chlorella* have been restricted by the existence of a rigid cell wall and the lack of an appropriate system for genetic analysis. So, induction of naked protoplast formation from *Chlorella* cells has been a matter of concern and interest (Atkinson et al. 1972).

The genus *Chlorella* includes heterogeneous species differing in morphological characteristics (Fott and Nováková 1969) and in biochemical and physiological characteristics (Kessler 1976). According to the recent taxonomical key proposed by Kessler (1978), *Chlorella* species are divided into two groups: one synthesizes secondary carotenoids under nitrogen deficiency and the other does not. The synthesis of secondary carotenoids was reportedly related to the existence of "sporopollenin" layer in the cell wall, which was shown to be responsible for the rigidity and indigestibility of *Chlorella* cell walls (Atkinson et al. 1972). In addition, *Chlorella* species are heterogeneous in the morphology and chemical composition of the cell wall (Soeder 1964) so that even strains without sporopollenin in their cell wall are also resistant to enzymatic digestion. The relationship between stability and structure or composition of *Chlorella* cell walls has never been fully clarified.

Recently, we investigated twelve strains of eight *Chlorella* species which lack a sporopollenin layer in their cell wall (secondary carotenoid-negative) and found that two strains (*C. ellipsoidea* C-87 and *C. saccharophila* C-211) can be induced to form osmotically labile protoplasts (Yamada and Sakaguchi 1981) by the treatment with some polysaccharide degrading enzyme mixtures.

In this study, we survey further phylogenetically related twelve *Chlorella* strains for protoplast formation by enzymatic digestion and compare ultrastructures of their cell walls. The relationship between stability and structure or composition of the *Chlorella* cell walls is discussed.

Materials and Methods

Experimental Cultures

All strains of *Chlorella* species were obtained from the algal culture collection of the Institute of Applied Microbiology, University of Tokyo. Cells of *C. ellipsoidea* C-87, C-102, and C-183; of *C. vulgaris* C-150, C-207, C-208, and C-209; and of *C. saccharophila* C-211 were cultured in a modified Bristol medium (Watanabe 1960) supplemented with 0.1% proteose peptone. Cells of *C. vulgaris* C-133 and C-135 were cultured in a modified Detmer medium (Watanabe 1960) and of *C. vulgaris* C-30 and C-169 in the *Closterium* medium (Ichimura 1973). The cultivations were carried out in flasks on a reciprocal shaker with a 16 h light at 3,000 lux and 8 h dark cycle for 4–5 days.

Induction of Protoplast Formation

Protoplast formation was induced with freshly harvested cells of *Chlorella* species as previously described (Yamada and Sakaguchi 1981): the cell suspension (2×10^8 cells/ml) in 25 mM phosphate buffer (pH 6.0) containing 0.6 M sorbitol/mannitol (1:1) and polysaccharide degrading enzymes such as (1) CMP; 4% cellulase Onozuka R-10 (Kinki Yakult MFG), 2% Macerozyme R-10 (kinki Yakult MFG), and 1% pectinase (*Asp. niger*, Sigma), (2) CP; 2% Cellulysin (Calbiochem) and 1% pectinase (*Asp. niger*, Sigma), or (3) DP; 2% Driselase (Kyowa Hakko Kogyo Co., LTD) and 1% pectinase (*Asp. niger*, Sigma) was incubated at 25° C in a water bath with gentle shaking. The formation of osmotically labile structure was examined by adding 0.1 ml of the suspension to

Offprint requests to: T. Yamada

0302-8933/82/0132/0010/$01.00

2.9 ml of water and by counting disrupted cells by a hemacytometer.

Electron Microscopy

Exponentially growing cells of *Chlorella* species were harvested and washed twice with 25 mM phosphate buffer (pH 6.5). The cells were fixed with 2% (w/v) glutaraldehyde in the same buffer for 4 h at room temperature and post-fixed with 1% OsO_4 in the same buffer for 2 h at 4° C. The fixed cells were dehydrated in a graded ethanol series, transferred to QY-1 (n-buthylglycidyl ether), and immersed in Epon (Millonig and Marrinozzi 1968). Thin sections were cut by a Sorval MT-1 microtome, and stained with 2% uranyl acetate for 30 min followed by a lead salts mixture (Millonig 1963) for 5 min. They were examined by a JEM-100B (JEOL) electron microscope.

Staining Procedures

The β-linked polysaccharides such as *cellulose* in the cell wall of *Chlorella* species were stained with Calcofluor White ST (Maeda and Ishida 1967) by the method of Nagata and Takebe (1970). Pectin was stained with Ruthenium Red (Sigma) according to Soeder (1963). The secondary carotenoids formation under nitrogen deficiency was tested according to Kessler and Czygan (1967).

Results

Protoplast Induction in Chlorella Strains

In addition to the strains of *C. ellipsoidea* C-87 and *C. saccharophila* C-211 from which protoplasts were formed (Yamada and Sakaguchi 1981), ten other strains of *Chlorella* (2, *C. ellipsoidea* and 8, *C. vulgaris*) were treated with cell wall digesting enzyme mixtures. *C. vulgaris* C-169 and C-135 among those tested formed protoplasts by the treatment with a mixture of cellulase Onozuka, Macerozyme, and pectinase (CMP), the strain C-135 forming protoplasts at a somewhat lower frequency (Table 1). The enzyme mixture CMP was most effective in all the protoplast formation in *Chlorella* strains except *C. saccharophila* C-211 whose cell walls were effectively digested only by Cellulysin-pectinase (CP). Driselase-pectinase (DP) was also effective for cell wall digestion in *C. vulgaris* C-169 (Table 1).

Table 1. Protoplast induction in *Chlorella* strains with some enzyme mixtures[a]

Strain		Frequency of protoplast formation		
		CMP	CP	DP
C. ellipsoidea[b]	C-87	90	10	0
C. vulgaris	C-135	20	10	0
	C-150	1	1	0
	C-169	80	3	30
C. saccharophila	C-211	1	26	2

[a] Cells of *Chlorella* strains (2×10^8 cells/ml) were treated with each enzyme mixture in 25 mM phosphate buffer (pH 6.0) containing 0.6 M sorbitol/mannitol. After incubation at 25° C for 12 h, osmotically labile structures were counted. CMP consisted of 4% cellulase Onozuka, 2% Macerozyme, and 1% pectinase, CP of 2% Cellulysin and 1% pectinase, and DP of 2% Driselase and 1% pectinase

[b] In addition to the strains listed the following strains were examined, however, without any protoplast formation: *C. ellipsoidea* C-102 and C-183, *C. vulgaris* C-30, C-133, C-207, C-208 and C-209

Table 2. Staining of cell wall with Calcofluor White and with Ruthenium Red and formation of secondary carotenoids in *Chlorella* strains

Cell wall type	Strain		Calcofluor[a] White	Ruthenium Red[b]	Secondary carotenoids
1.	*C. ellipsoidea*	C-102	++	+	–
	C. vulgaris	C-209	+	±	–
2.	*C. ellipsoidea*	C-87	++	±	–
	C. ellipsoidea	C-183	++	+	–
	C. vulgaris	C-169	++	+	–
	C. vulgaris	C-208	+	±	–
	C. saccharophila	C-211	++	–	–
3.	*C. vulgaris*	C-30	+	+	–
	C. vulgaris	C-133	+	+	–
	C. vulgaris	C-135	+	+	–
	C. vulgaris	C-150	+	+	–
	C. vulgaris	C-207	+	+	–

[a] ++ Intense blue fluorescence from the whole surface; + haloing weak fluorescence

[b] + Intense red; ± pink; – no red colour

Comparison of Cell Wall Structures among Chlorella Strains

The results of cell wall digestion experiments suggested that the structure and/or composition of *Chlorella* cell walls might be considerably different from one strain to the other. Among the same species, some strains formed protoplasts and others did not. Furthermore, the attitude towards digestion enzymes was different even among the strains forming protoplasts. To compare ultrastructure of the cell walls and to elucidate the relationship between the structure and the sensitivity to enzymatic digestions of the cell wall, electron microscopic observations were carried out. As shown in Fig. 1, three types of the cell wall structures independent of the *Chlorella* species were revealed: Type 1, the cell wall composed of two layers; the inner bulky electron-transparent or microfibrillar layer and the outer trilaminar layer (Fig. 1 a, b); Type 2, the cell wall composed of also two major layers but the outer layer is not trilaminar (Fig. 1 c – g); and Type 3, the cell wall composed of only one microfibrillar layer (Fig. 1 h–l). Obvious intraspecific heterogeneity of cell wall structures existed in *C. vulgaris* and *C. ellipsoidea;* there were some accessory structures on the cell wall of *C. vulgaris* C-102. Cell walls of the three strains C-87, C-169, and C-211 which formed protoplasts belong to Type 2, which indicates that the cell wall of Type 2 would be composed of enzymatically digestible components or constructed of weaker protective structures. As for the strain C-135, another protoplast-forming strain, the cell wall was of Type 3 but the microfibrillar network was very sparce, and at places, the cell wall was very thin (Fig. 1 j).

Chemical Composition of Chlorella Cell Walls

Cell walls of each type were stained with Calcofluor White ST for β-linked polysaccharides such as cellulose and with

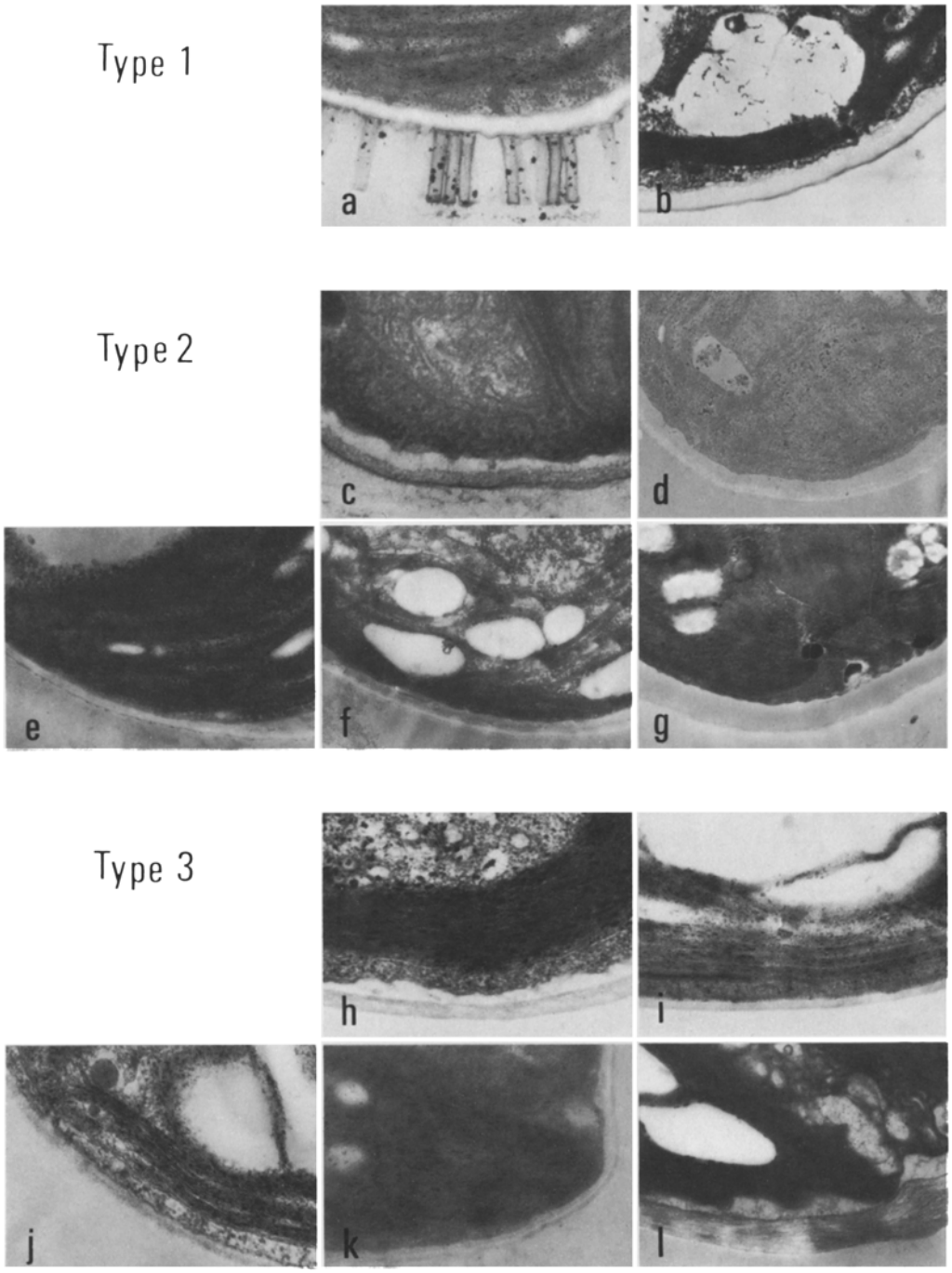

Fig. 1a–l. Electron micrographs showing thin sections of the cell wall of *Chlorella* strains. (**a**) *C. ellipsoidea* C-102, (**b**) *C. vulgaris* C-209, (**c**) *C. ellipsoidea* C-87, (**d**) *C. vulgaris* C-169, (**e**) *C. ellipsoidea* C-183, (**f**) *C. vulgaris* C-208, (**g**) *C. saccharophila* C-211, (**h**) *C. vulgaris* C-30, (**i**) *C. vulgaris* C-133, (**j**) *C. vulgaris* C-135, (**k**) *C. vulgaris* C-150, and (**l**) *C. vulgaris* C-207. × 30,000

Ruthenium Red for pectin. Formation of secondary carotenoids under nitrogen deficient conditions was also tested to examine the existence of sporopollenin (Atkinson et al. 1972). Results are shown in Table 2. It is evident that some relation between structure and chemical composition exists; the cell walls of Type 2 contained abundant substances stainable with Calcofluor and comparably small amounts of pectin, on the other hand, those of Type 3 were intensely stained with Ruthenium Red and somewhat weakly with Calcofluor which reflects the existence of much pectin and a small amount of β-linked polysaccharides. No strains showed any evidence of the secondary carotenoid formation indicating the absence of sporopollenin in the outer layer of the cell wall.

Discussion

So far, reports concerning the protoplast induction in *Chlorella* have been restricted to *C. saccharophila* (Braun and Aach 1975; Aach et al. 1978), *C. vulgaris* (Berliner 1977) and *C. ellipsoidea* and *C. saccharophila* (Yamada and Sakaguchi 1981). The three species are closely related to each other; some strains called *C. ellipsoidea* earlier have been included in

C. vulgaris and the taxon of *C. ellipsoidea* itself has been merged into *C. saccharophila* (Fott and Nováková 1969). By a recent taxonomical method that bases on DNA hybridization data, *C. vulgaris* and *C. saccharophila* have been put in one group, "the *C. vulgaris* group" (Kerfin and Kessler 1978). Therefore, strains of the three species are similar to each other and share some common morphological, biochemical, and physiological characteristics (Fott and Nováková 1969).

In this study, we treated 12 strains of these three *Chlorella* species with polysaccharide degrading enzyme mixtures and found that 4 strains form protoplasts. The sensitivity of cell wall to enzymatic digestion was related to the structure and the composition of cell wall; the digestible cell wall was of Type 2 containing much substances stainable with Calcofluor (β-linked polysaccharides) and a little pectin. The cell wall of Type 2 was composed of the outer non-trilaminar layer and the inner microfibrillar layer. In the course of protoplast formation in *C. ellipsoidea* C-87, it was found that the inner layer was digested during the first few hours and then the outer thin layer was broken and peeled off (Yamada and Sakaguchi 1982). The outer rigid layer would protect the inner bulky microfibrillar layer which is probably composed of cellulose, since it was easily digested by cellulase. As for the Type 1 cell wall, the outer trilaminar layer is strikingly similar to that reported by Atkinson et al. (1972) although no evidence for the production of secondary carotenoids was obtained in the strains C-102 and C-209. The outer layer might, like the sporopollenin layer, be responsible for inability to form protoplasts. Indeed, under normal culture conditions, the cell wall of Type 1 persists long after a liberation of autospores. In the cell wall of Type 3, the outer layer was absent and the structure of cell wall was apparently homogeneous. On the other hand, pectin was strongly detected and β-linked polysaccharides were only weakly so in the cell wall. It indicates some heterogeneous composition of the cell walls of Type 3; the compositional heterogeneity might be a cause of resistance to the enzymatical digestions. One exception was the case of *C. vulgaris* C-135, whose cell wall was of Type 3 but digestible with enzymes (Table 1) at a somewhat lower frequency. Since the cell wall of C-135 often varied in thickness and consisted of a sparce network (Fig. 1j), there may be some localities sensitive to the enzymatic attack. The result obtained here is somewhat complicated; the three types of cell wall did not accord with the three species. Though most strains of *C. vulgaris* tested in this study belong to the Type 3, some do to Type 1 or Type 2. It is also the case for strains of *C. ellipsoidea*; the strain C-102 possessed a unique cell wall structure, the trilaminar outer layer and the projections on the surface of its cell wall (Fig. 1a). This apparent discrepancy remains to be solved; if the cell wall structure is considered as a taxonomically significant character, the assignment of the 12 strains in this study should be re-examined.

Since some of the protoplasts obtained here could regenerate a cell wall and grow in a regeneration medium (Yamada and Sakaguchi 1982), the possibility of intraspecific and interspecific protoplast fusion of *Chlorella* is now expected to be realized.

References

Aach HG, Bartsch S, Feyen V (1978) Studies on *Chlorella* protoplasts: Demonstration of the protoplastic nature and the regeneration of the cell wall. Planta 139:257–260

Atkinson AW, Jr, Gunning BES, John PCL (1972) Sporopollenin in the cell wall of *Chlorella* and other algae: Ultrastructure, chemistry, and incorporation of ^{14}C-acetate, studied in synchronous cultures. Planta 107:1–32

Berliner MD (1977) Protoplast induction in *Chlorella vulgaris*. Plant Sci Lett 9:201–204

Braun E, Aach HG (1975) Enzymatic degradation of the cell wall of *Chlorella*. Planta 126:181–185

Fott B, Nováková M (1969) A monograph of the genus *Chlorella*. The fresh water species. In: B Fott (ed) Studies in phycology. Academia, Prag, pp 10–74

Ichimura T (1973) Sexual cell division and conjugation-papilla formation in sexual reproduction of *Closterium strigosum*. In: K Nishizawa (ed) Proceedings of the seventh International Seaweed Symposium. University of Tokyo Press, Tokyo, pp 208–214

Kerfin W, Kessler E (1978) Physiological and biochemical contributions to the taxonomy of the genus *Chlorella*. XI. DNA hybridization. Arch Microbiol 116:97–103

Kessler E (1976) Comparative physiology, biochemistry, and the taxonomy of *Chlorella (Chlorophyceae)*. Plant Syst Evol 125:129–138

Kessler E (1978) Physiological and biochemical contributions to the taxonomy of the genus *Chlorella*. XII. Starch hydrolysis and a key for the identification of 13 species. Arch Microbiol 119:13–16

Kessler E, Czygan F-C (1967) Physiologische und biochemische Beiträge zur Taxonomie der Gattungen *Ankistrodesmus* und *Scenedesmus*. I. Hydrogenase, Sekundär-Carotinoide und Gelatine-Verflüssigung. Arch Mikrobiol 55:320–326

Maeda H, Ishida N (1967) Specificity of binding of hexapyranosyl polysaccharides with fluorescent brightener. J Biochem 62:276–278

Millonig G (1963) A modified procedure for lead staining of thin sections. J Biophys Biochem Cytol 11:736–739

Millonig G, Marinozzi V (1968) Fixation and embedding in electron microscopy. In: R Barer, VE Cosslett (eds) Advances in optical and electron microscopy, Vol 2. Academic Press, London, pp 251–341

Nagata T, Takebe I (1970) Cell wall regeneration and cell division isolated tobacco mesophyll protoplasts. Planta 92:301–308

Soeder CJ (1963) Weitere zellmorphologische und physiologische Merkmale von *Chlorella*-Arten. In: Studies on microalgae and photosynthetic bacteria. Japanese Society of Plant Physiologists, Tokyo, pp 21–34

Soeder CJ (1964) Elektronenmikroskopische Untersuchungen an ungeteilten Zellen von *Chlorella fusca* Shihira et Krauss. Arch Mikrobiol 47:311–324

Yamada T, Sakaguchi K (1981) Protoplast induction in *Chlorella* species. Agric Biol Chem 45:1905–1909

Yamada T, Sakaguchi K (1982) Electron microscopic studies on *Chlorella ellipsoidea* protoplast formation. J Gen Microbiol (in press)

Watanabe A (1960) List of algal strains in collection at the Institute of Applied Microbiology, University of Tokyo. J Gen Appl Microbiol 6:283–292

Received December 4, 1981/Accepted April 5, 1982