STUDIES ON PROTOPLASTS OF CHLORELLA:

PREPARATION AND UTILIZATION

(Chlorella の プロトプラスト調製とその利用に関する研究)

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TAKASHI YAMADA

CONTENTS

PREFACE

A group of green algae represented by the genus Chlorella (Chlorococcales) is ubiquitous in fresh water, in marine water, and in soil on the Earth. Because of its simple structure, simple life cycle, rapid growth on various conditions, and modest nutritional requirements, Chlorella has, over a long period of time, served as a model organism in plant physiological and biochemical researches; several fundamentally important findings in these disciplines such as the Calvin-Benson cycle of photosynthesis (Calvin and Bassham, 1962) and the general pathway of chlorophyll biosynthesis (Granik, 1979) were made with this organism.

Besides these interests in basic researches, Chlorella cells have also been an interesting and important experimental material in applied and industrial microbiology. With the gradual advent of general public concern about problem of food production, energy supply, and pollution, unicellular algae of the Chlorella type have been intensively studied and used in a variety of practical applications in agriculture and technology, i.e. as protein-rich food or feed (in terms of overall protein yield, the algal culture reportedly gives 6-30 times as much protein as leguminous plants according to Vincent, 1971) and for the production of biomass for conversion to energy.

The ability of Chlorella to degrade a variety of inorganic and

organic compounds (Wiessner, 1970) has aroused interests in purification of sewage and waste (Galloway and Krauss, 1963).

In the microbiological technology or biochemical engineering field, Chlorella has frequently used as a model organism in researches on mass-cultivation, synchronous culture, and planning of culture installations (Tamiya et al., 1953; Myers and Graham, 1959; Tamiya, 1964). On the basis of the knowledge obtained through studies with Chlorella, various types of indoor cultivation tanks, outdoor ditches, and ponds were constructed.

In spite of many attractive and useful characters of Chlorella as mentioned above, two disadvantages of this alga have seriously restricted further progress of researches in both basic and applied microbiology: they are (1) the existence of an extraordinarily rigid and indigestible cell wall and (2) the lack of sexual process of reproduction in the cell cycle. The former prevents not only extraction and isolation of the cellular materials and some genetic manipulations such as cell fusion, organelle transplantation, and transformation with external DNAs but also utilization of the cells as food or feed; fresh, dried or solvent-extracted Chlorella cells are hardly digested at all by humans (Mitsuda, et al., 1969) and by other non-ruminant animals (Meffert and Pabst, 1963), although the isolated algal protein is easily digested by non-ruminants (Mitsuda, et al., 1969). The latter makes genetic studies difficult; in fact, nothing has been known concerning genetics and molecular

biology about Chlorella. On the other hand, Chlamydomonas reinhardii, a green alga related to Chlorella has been an excellent organism for genetic investigations and lots of knowledge have been obtained by virtue of its sexuality (Lewin, 1976).

To overcome disadvantages and to make Chlorella cells a more expedient subject in basic and applied microbiological researches, several techniques for homogenization or fractionation of the cells were introduced: those were the mechanical methods (Theriault, 1965), the chemical methods (Mitsuda, 1965; Hedenskog, et al., 1969), and enzymatic digestion (Northcote, et al., 1958; King and Shefner, 1962; Wiseman, 1969). Although the gastric juice from snail (Helix pomatia) attacked isolated cell wall of Chlorella pyrenoidosa (Northcote, et al., 1958), it was found to be ineffective against whole cells (King and Shefner, 1962). Wiseman (1969) could not find any enzymatic method that was active for the whole cells.

If protoplast (naked cell) formation can be induced in Chlorella some intact cellular components such as plastid and plastid DNA as well as nucleus and nuclear DNA might be isolated, which makes it possible to advance Chlorella biology at a molecular level. Some protoplast fusion experiments would produce a system for genetic analysis in this strict celibate organism. By establishing a system for transformation with protoplasts,

molecular cloning of some useful genes in Chlorella as a host, solely using solar energy, may be realized. Thus induction of protoplast formation in Chlorella cells has been a matter of concern and interest for a long period of time (Atkinson, et al., 1972).

The present work started in order to open up new possibilities in the directions of researches on Chlorella which is outlined above. I wish that the results presented here contribute to the progress in both basic and applied microbiology.

GENERAL INTRODUCTION

The green algal genus Chlorella includes very heterogeneous species which differ in morphological characteristics (Fott and Nováková, 1969), in biochemical and physiological characteristics (Kessler, 1976), and in the GC content of DNA from 43 to 79 % (Hellmann and Kessler, 1974). Cell walls of Chlorella strains are also considerably different from each other in morphology and chemical compositions (Soeder, 1963). In some species, it was shown that the existence of sporopollenin layer in the cell wall is responsible for the rigidity and indigestibility of Chlorella cell walls (Atkinson et al., 1972). Most Chlorella strains without sporopollenin in their cell walls, however, are also resistant to enzymatic digestion. The relationship between rigidity and structure or chemical composition of Chlorella cell walls has never been fully clarified.

The matter of first concern in this work was to develop a method to induce protoplast formation from Chlorella species so that the heterogeneity in structure or chemical composition of Chlorella cell walls could be known, and that the relationship between rigidity or indigestibility and structure or chemical composition of Chlorella cell walls could be clarified.

In the second place of this work, the usefulness and advantages of Chlorella protoplasts in several kinds of researches was demonstrated: (1) the ability of protoplasts to regenerate

a cell wall and to grow, (2) the occurrence of protoplast fusion, and (3) the possibility to isolate several intact cellular components such as chloroplasts and chloroplast DNAs from the protoplasts.

Another matter of concern in this work is the molecular biological aspects of Chlorella chloroplast DNA which was, for the first time, revealed through the effective extraction of the DNAs from protoplasts. Its fundamental importance could be explained as below. Chlorella cells as well as other photoautotrophic eukaryotic organisms harbour some cellular organelles such as chloroplasts and mitochondria, each of which contain its own genetic machinery. Chloroplasts, especially playing a fundamental role in cellular energy metabolism, were suggested to possess at least some of genetic information involved in photosynthesis (Ciferri, 1978). Existing evidence from several plants and algae indicates that a single, double-stranded circular DNA molecule, the chromosome, contains the coding potencial of the organelle. So far, chloroplast DNAs have been isolated from several plants and algae, and their characteristics have been compared with each other (Herrmann and Possingham, 1980). Special aspects of algal chloroplast DNAs are: (1) the wide range of size distribution, from 5.6×10^7 dalton for Codium fragile (Hedberg et al., 1981) to $1.1 \sim 1.5 \times 10^9$ dalton for Acetabularia (Padmanabhan and Green, 1978); (2) the unique physical arrangement of the DNA molecule such as the existence of inverted or tandemly repeated sequences and

some intervening sequences in the 23S rRNA gene (Rochaix, 1981); and (3) the occurrence of methylated nucleotides (Royer and Sager, 1979).

Thus, it is of great interest to investigate the physical properties and the molecular arrangement of chloroplast DNAs from such a familiar and ubiquitous alga as Chlorella. Information from the chloroplast DNAs may elucidate an evolutional relationship of this alga to higher plants or several other algal groups.

The nature of cooperation between chloroplast and nucleus or mitochondria, which is a fundamental problem in biology, may be also learned after mapping on the chloroplast DNA some specific genes such as those coding for rRNAs, tRNAs, ribosomal proteins, the large subunit of ribulose-1,5-bisphosphate carboxylase (RuBPCase), etc, and studing their expression mechanisms. Characterization of chloroplast genes involved in photosynthesis will consequently open up a way to expand photosynthetic activities through molecular biological techniques in plant and microbiological worlds.

In this thesis, Chapter I demonstrates (1) induction of protoplast formation in two Chlorella strains (C. ellipsoidea C-87 and C. saccharophila C-211) among twelve strains tested, (2) regeneration of whole cells from C. ellipsoidea protoplasts, and (3) occurrence of some protoplast fusion. Chapter II shows an existence of three types of cell walls in the phylogenetically related Chlorella strains without secondary carotenoids in the cell wall. The chapter also shows different behaviours of

these three types of cell walls towards the enzymatic digestion and the chemical staining tests. A simple method is offered to distinguish Chlorella strains that can be induced to form protoplasts from the strains with a rigid cell wall and not forming protoplasts. In Chapter III, the process of protoplast formation from C. ellipsoidea C-87 and the cell wall structure of this strain is described in detail. It is revealed that the rate-limiting barrier against the protoplast formation is the outermost thin layer of the cell wall which is probably composed of pectin. Regeneration of the cell wall and the subsequent growth of the protoplasts are also confirmed.

From protoplasts of C. ellipsoidea C-87 thus formed, several cellular components were isolated. Chapter IV demonstrates the isolation and characterization of intact chloroplasts and chloroplast DNA. The chloroplast DNA is characterized in its circularity, contour length, molecular weight, GC-content, restriction fragment patterns, and the absence of DNA methylation.

In Chapter V, the molecular arrangement of the chloroplast DNA is charactrized; the DNA contains a pair of large inverted repeat sequences, a small single copy region, and a large single copy region. The chloroplast rRNA genes (16S and 23S) are mapped in the small loop end of the inverted repeats on the chloroplast DNA. In this chapter, some differences in molecular arrangement of Chlorella chloroplast DNA from that of other algae such as Chlamydomonas or Euglena are also discussed.

In Chapter VI, as conclusion of this work, several significant matters are discussed: those are nature of protoplast-forming *Chlorella* strains, efficiency of the protoplast formation, usefulness of *Chlorella* protoplasts in industry and in basic researches, and molecular biological aspects of *Chlorella* chloroplast DNA.

CHPTER I

Preparation of Protoplasts from *Chlorella* Species

INTRODUCTION

A rigid and indigestible cell wall of *Chlorella* has often been a problem against studies on *Chlorella*. In some strains, the protector against an enzymatic attack is a trilaminar outer layer containing sporopollenin, an extraordinarily resistant polymer of carotenoids (Atkinson *et al.*, 1972). The cell walls of other strains which lack the trilaminar component have a wide variety of resistance against enzymatic digestions. Although most of them were also generally indigestible, there are two examples of *Chlorella* protoplast formation so far reported in literature. In one case, it took nearly four days to form spheroplasts of *C. saccharophila* by a treatment with a polysaccharide degrading enzyme mixture (Braun and Aach, 1975). If the resistant outermost layer of *C. saccharophila* is scratched by sea sand, the cell wall is much more rapidly degraded by cellulase (Aach *et al.*, 1978). On the other hand, protoplasts of *C. vulgaris* were formed with 0.4 % Cellulysin during several hours (Berliner, 1977). In that case, the protoplasts were extruded through breaks in the cell wall and not by dissolution of the wall.

I describe here two strains of *Chlorella* which are unable to produce secondary carotenoids (Atkinson *et al.*, 1972) and lack the sporopollenin layer in the cell wall can be easily induced to form protoplasts by a treatment with some enzyme mixtures.

MATERIALS AND METHODS

Experimental cultures. Strains examined and their sources were as follows: *Chlorella ellipsoidea* Gerneck (C-27 and C-87), *C. luteoviridis* Chodat (C-97), *C. prototechoides* Krüger (C-99 and C-202), *C. prototechoides* Krüger var. *mannophila* Shihira et Krauss (C-206), *C. pyrenoidosa* Chick (C-101) and *C. saccharophila* Krüger (C-211) from the algal culture collection of the Institute of Applied Microbiology, Univ. of Tokyo; *C. sorokiniana* Shihira et Krauss (211-8k, 211-40a) from Prof. E. Kessler (Institut für Botanik und Pharmazeutische Biologie der Universität); *C. vulgaris* Beijerinck (11h) from Prof. S. Miyachi (the Institute of Applied Microbiology, Univ. of Tokyo); *C. vulgaris* Beijerinck (CBSC) from Carolina Biological Supply Company, North Carolina.

These twelve strains of *Chlorella* species were selected because they do not form secondary carotenoids under nitrogen deficiency (Kessler *et al.*, 1963) so that they lack the outer trilaminar sporopollenin layer in the cell wall which is a resistant polymer of carotenoids and is responsible for indigestibility (Atkinson, *et al.*, 1972).

The cells were cultured in a modified Bristol medium (MBM) (Watanabe, 1960) supplemented with 0.1 % proteose peptone (MBMP) at 25°C with a 16h light at 3,000 lux and 8h dark cycle for 4 to 5 days.

Digestion of cell wall with enzymes. Cells were harvested and

washed with 25 mM phosphate buffer (pH 6.0) and suspended in the same buffer containing 0.6 M sorbitol/mannitol (1:1) and some of the following polysaccharide-degrading enzymes: 4% cellulase Onozuka R-10 (Kinki Yakult MFG), 2 % Macerozyme R-10 (Kinki Yakult MFG), 1 % pectinase (Asp. niger, Sigma), 2 % hemicellulase (Asp. niger, Sigma), 1 % chitinase (Streptomyces griceus, Sigma), 2 % Driselase (Kyowa Hakko Kogyo Co., LTD) and 2 % Cellulysin (Calbiochem).

The cell suspension (2×10^8 cells/ml) was incubated in the light at 25°C in a shaker water bath with gentle shaking. The formation of osmotically labile structures was monitored by adding 0.1 ml of the suspension to 2.9 ml of water and by counting disrupted cells with a hemacytometer. The staining of the cells with Calcofluor White was carried out according to Nagata and Takebe (1970) for 30 min at room temperature.

Electron microscopy. Portions of the suspension of the cells in the enzyme mixture were removed at intervals and centrifuged at 1,000 x g at 4°C. After washing twice with 25 mM phosphate buffer that contained 0.6 M sorbitol/mannitol, cells were fixed with 3 % (wt/vol) glutaraldehyde in the same buffer for 2 h at room temperature and post-fixed with 1 % O_sO_4 in the same buffer for 1 h at 4°C. For scanning electron microscopy, the fixed cells were dehydrated in a graded ethanol series and critically point dried. A small portion of the dried cells was attached to a conductive stub and sputter-coated with gold in a Eiko JB-3 Ion Coater. Samples were examined with a JEM-100CX (JEOL) scanning

electron microscope. For transmission electron microscopy, the fixed cells were embedded in 2 % agar, dehydrated as above, transferred to QY-1 (n-butylglycidyl ether), and immersed in Epon (Millonig and Marinozzi, 1968). Thin sections were cut by a Sorval MT-1 microtome, and stained with 2 % uranylacetate for 30 min followed by a lead salts mixture (Millonig, 1963) for 5 min. They were examined with a JEM-100B (JEOL) electron microscope.

Viability of protoplasts. The protoplasts were embedded in 5 ml isotonic soft agar (0.6 %) and plated on both a normal MBMP agar (2 %) supplemented with 0.1 % yeast extract (MBMPY) and a MBMPY agar containing either 0.6 M sorbitol/mannitol or 20 % sucrose. Plates were incubated at 25°C in the light (3,000 lux). The difference in the number of colonies on the two media after 2 weeks was considered to represent the number of viable protoplasts.

RESULTS

Preparation of protoplasts.

Effective protoplast formation was observed in the cells of *C. ellipsoidea* C-87 and *C. saccharophila* C-211 when the former was treated with cellulase, Macerozyme, and pectinase, and the latter with Cellulysin and pectinase (Table I-1). An addition of water ruptured their protoplasts; the light green content leaked out from the remaining cell structure. The other ten strains of *Chlorella* species did not form osmotically sensitive structures by

Table I-1. Protoplast formation in *Chlorella* species with some enzyme mixtures*

Strains		Protoplast frequency (%)		
		CMP	DP	CP
C. ellipsoidea	IAM C-27	3	0	2
	IAM C-87	90	0	10
C. luteoviridis	IAM C-97	1	0	0
C. prototechoides	IAM C-99	0	0	0
	IAM C-202	0	0	0
var. *mannophila*	IAM C-206	0	0	0
C. pyrenoidosa	IAM C-101	0	0	0
C. saccharophila	IAM C-211	1	2	26
C. sorokiniana	211-8k	0	0	0
	211-40a	0	0	0
C. vulgaris	11h	1	0	2
	CBSC	0	0	0

* Cells of *Chlorella* species (2-5 × 10^8/ml) were treated with each enzyme mixture in 25 mM phosphate buffer (pH 6.0) containing 0.6 M sorbitol/mannitol. After an incubation at 25°C for 24 hr, number of osmotic labile structures was counted. CMP consisted of 4 % cellulase, 2% Macerozyme, and 1 % pectinase. DP of 2 % Driselase, and 1 % pectinase. CP of 2 % Cellulysin and 1 % pectinase.

Table I-2. Protoplast formation in C. ellipsoidea with some enzyme mixtures*

Enzymes	Protoplast frequency (%)
Cellulase (4%) + Macerozyme (2%) + pectinase (1%)	90
Cellulase (4%) + Macerozyme (2%)	0
Cellulase (4%) + pectinase (1%)	0
Macerozyme (2%) + pectinase (1%)	0
Cellulase (4%) + hemicellulase (2%) + pectinase (1%)	0
Chitinase (1%) + hemicellulase (2%) + pectinase (1%)	0
Driselase (2%) + pectinase (1%)	0
Cellulysin (2%) + pectinase (1%)	10

* Cells of C. ellipsoidea (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 (1 : 1). After incubation at 25°C for 24 h, number of osmotically labile structures was counted under a microscope.

treatments for 24 h with any combinations of the cell wall digestion enzymes shown in Table I-1.

About 90 % of C. ellipsoidea C-87 cells converted protoplasts after 24 h by the treatment with 4 % cellulase, 2 % Macerozyme and 1 % pectinase (CMP), however, lack of any one of these three enzymes resulted in no cell wall degradation (Table I-2). Although a small portion (10 %) of the cells were induced to form protoplasts by the combination of 2 % Cellulysin and 1 % pectinase, other enzymes such as hemicellulase, chitinase and Driselase showed no effects. The time course of the cell wall digestion in C. ellipsoidea C-87 by the CMP treatment is shown in Fig. I-1. About 50 % of the cells became osmotic-labile after 4 h incubation and 80 % of the cells after 8 h incubation. The protoplast formation was also monitored by Calcofluor staining of the cell wall. Whole cells and cell wall fragments stained with Calcofluor White showed an intense light blue fluorescence when irradiated with UV. Protoplasts showed no blue but red fluorescence due to chlorophylls.

Electron microscopic observations.

Scanning electron micrographs showed that the intact vegitative cells of C. ellipsoidea C-87 are ellipsoidal (3.5 x 4 μm) with slightly furrowed surface (Fig. I-2a). Fig. I-2b shows a thin section of the intact vegitative cell; there are a saucer-shaped chloroplast, a large nucleus, vacuoles, and mitochondria. The

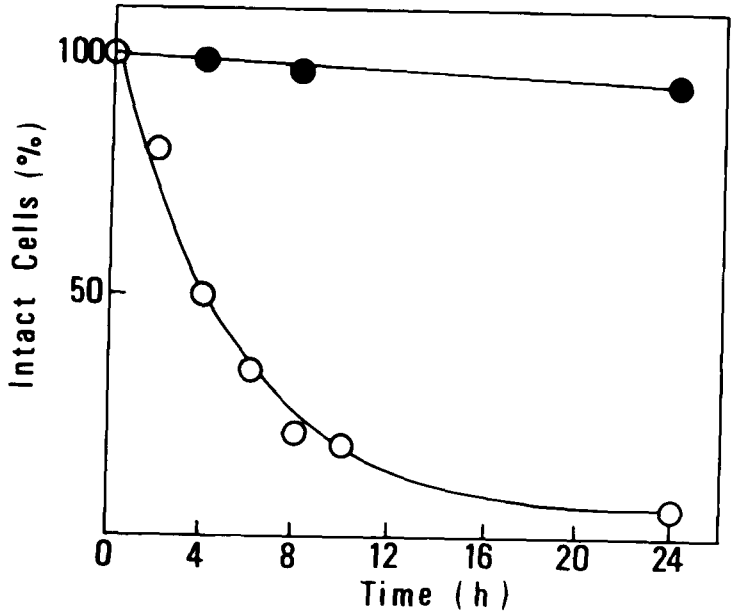


Fig. I-1. Time course of the protoplast formation. Cells of C. ellipsoidea C-87 were suspended (2 x 10^8 cells/ml) in the induction medium composed of 4 % cellulase Onozuka, 2 % Macerozyme and 1 % purified pectinase (pH 6.0). After the incubation at 25°C, small portions (0.1 ml) were pipetted out and diluted with water (o) or with 0.6 M sorbitol/mannitol (●). Number of osmotically sensitive structures was counted by a hemacytometer.

cell wall consists of two regions (Fig.I-2c); the outer narrow region is electron dense (10 nm in width) and the inner electron opaque region is much more bulky and microfibrillar (width: 100 nm to 200 nm). The wall structures were also observed in the maturing autospores (Fig. I-2d); they are enveloped by the mother cell wall whose inner region having become thinner as the cell walls of autospores develop. No trilaminar outer component of sporopollenin (Atkinson et al., 1972) was observed at any stages of the cell cycle. Figs. I-3a and b show typical protoplasts. They are spherical, 4.5 μm in diameter with a somewhat rugged surface, and without any structures of cell wall (Fig. I-3c). The protoplasts were frequently observed to adhere to each other (Fig. I-3d) as reported by Braun and Aach (1975) and by Berliner (1977).

Regeneration of cell wall and growth of protoplasts.

The protoplasts suspended in MBMP containing 0.6 M sorbitol/ mannitol could after 3-4 day- incubation at 25°C in the light (3,000 lux) regenerate the cell wall which was stained with Calcofluor. When the protoplasts were embedded in 0.6 % soft agar MBMPY containing 20 % sucrose, up to 30 % of protoplasts could grow and form colonies after 2-week incubation at 25°C in the light.

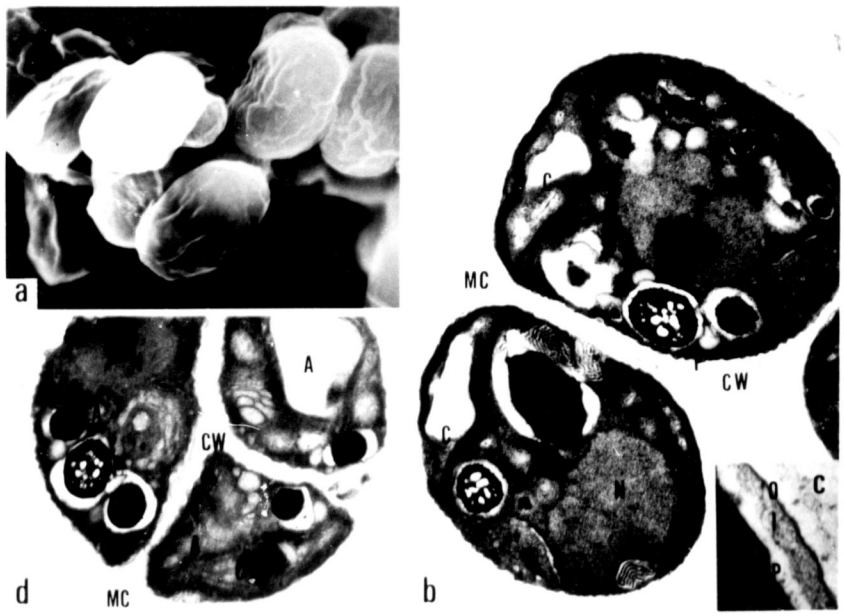


Fig. I-2. Electron micrographs showing intact cells of C. ellipsoidea C-87. (a) Scanning electron micrograph (x 7,000). (b) Thin section of two vegetative cells released from a mother cell wall (x 20,000). (c) Thin section of cell wall (x 50,000). (d) thin section of autospores (x 20,000). A, autospore; C, chloroplast; CW, cell wall; I, inner region of cell wall; M, mitochondrion; MC, mother cell wall; N, nucleus; O, outer region of cell wall; P, plasma membrane.

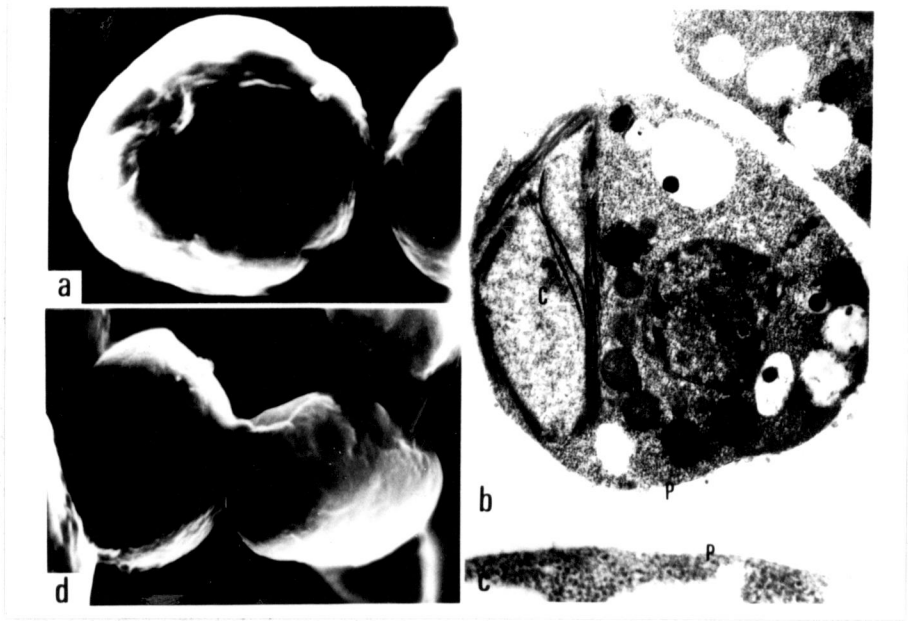


Fig. I-3. Electron micrographs showing the protoplasts of C. ellipsoidea. (a) Scanning electron micrograph (x 15,000). (b) Thin section of a protoplast. C, chloroplast; M, mitochondrion; N, nucleus; P, plasma membrane (x 20,000). (c) Thin section of the surface of a protoplast (x 50,000). (d) Scanning electron micrograph showing two protoplasts adhering to each other (x 10,000).

DISCUSSION

According to Soeder (1964), different species and strains of *Chlorella* vary with respect to the morphology and composition of their cell walls. Sporopollenin in the cell wall is reportedly related to the ability to produce secondary carotenoids (Atkinson, *et al.*, 1972). In this study, I selected 12 strains of *Chlorella* which do not produce secondary carotenoids (Kessler *et al.*, 1963). It was found that two of them, especially *C. ellipsoidea* C-87, could easily form protoplasts. The cell wall of *C. ellipsoidea* C-87 consists of two components; a fairly distinct but non-trilaminar outer zone and an inner microfibrillar zone (Fig. I-2c). They were degraded by an enzyme mixture containing cellulase, Macerozyme, and pectinase. Since the cellulase Onozuka (derived from *Trichoderma viride*) and the Macerozyme (derived from *Rhizopus* sp.) are crude enzyme mixtures containing several kinds of polysaccharide degrading activities (Cocking, 1972), agents necessary to digest the cell wall are yet unknown. Pectinase is, however, essential for the degradation because only the presence of highly purified pectinase (Sigma) resulted in the effective protoplast formation.

Takeda and Hirokawa (1978) reported that the cell wall of *C. ellipsoidea* C-27 consists of two components; alkali-soluble "hemicellulose" composed of rhamnose, xylose, arabinose, mannose, and galactose and alkali-insoluble "rigid wall" composed of

glucosamine. I showed that the mixture of hemicellulase, chitinase, and pectinase did not produce any osmotically sensitive structures from the strain C-87 (Table I-2) and that the strain C-27 was resistant to the treatment with CMP (Table I-1). Therefore, chemical compositions and/or structures of the cell walls of the two may be considerably different.

Since C-87 protoplasts can regenerate their cell wall and form colonies on a stabilized medium, some cell fusion experiments for genetic analyses of this strain would be made possible; in fact, a spontaneous protoplast fusion was frequently observed (Fig. I-3d). An easy isolation of some cellular components such as nuclei, chloroplasts, mitochondria or DNAs from the protoplasts would be also possible.

SUMMARY

Twelve strains of Chlorella which lack the sporopollenin layer in their cell wall were treated with polysaccharide degrading enzyme mixtures. Osmotically labile protoplasts were obtained from two of them (C. ellipsoidea C-87 and C. saccharophila C-211). The absence of the cell wall was demonstrated by the Calcofluor stain and by electron microscopies. Some protoplasts adhered to each other; it seemed like a cell fusion. Protoplasts of C. ellipsoidea C-87 were able to regenerate the cell wall and to grow on a regeneration medium.

CHAPTER II

Comparative Studies on Chlorella Cell Walls: Possibility of Protoplast Formation

INTRODUCTION

The green algal 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 fully clarified.

In Chapter I, I investigated twelve strains of eight Chlorella species which lack a sporopollenin layer in their cell wall (secondary carotenoids -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. According to Fott and Nováková (1969), the old taxon of C. ellipsoidea itself has been merged into C. saccharophila and some strains called C. ellipsoidea earlier have been included in C. vulgaris. Recently, the two species, C. saccharophila and C. vulgaris were shown to be closely related by DNA-DNA hybridization data (Kerfin and Kessler, 1978). Therefore, strains of Chlorella which are possible to form protoplasts seem to be restricted within a closely related group.

In this study, I survey further phylogenetically related twelve strains of three Chlorella species, C. ellipsoidea, C. saccharophila, and C. vulgaris 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. In addition to two strains used in Chapter I (C. ellipsoidea C-87 and C. saccharophila C-211), two strains of C. ellipsoidea (C-102 and C-183) and eight strains of C. vulgaris (C-30, C-133, C-135, C-150, C-169, C-207, C-208, and C-209) 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) supple-

mented 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 as described in Chapter I.

Preparation of protoplasts. Protoplast formation was induced with freshly harvested cells of Chlorella species as described in Chapter I.

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, dehydrated, transferred to QY-1, and immersed in Epon as described in Chapter I. Ultrathin sections were prepared and observed as in Chapter I.

Staining procedures. The β-linked polysaccarides 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). Calcofluor White ST is a bleaching powder or a brightener (disodium salt of 4,4′-bis[4-anilino-bis-diethyl amino-S-triazin-2-ylamino]-2,2′-stilbene-disulfonic acid). When combined with the dye and irradiated with UV light, hexapyranose polymers with β-configuration such as cellulose or chitin display intense blue fluorescence, while those of α-configuration such as starch or glycogen show only insignificant fluorescence. Cells were treated with a saturated solution of Calcofluor in 0.1 M Tris-HCl buffer (pH 7.2) for 30 min at room temperature

and washed five times with the same buffer before examination with a Nikon Optiphot XF-EF epifluoromicroscope. Pectin was stained with Ruthenium Red (Sigma) according to Soeder (1963). Cells were treated with a 0.2 % ammonia-solution of Ruthenium Red for up to four hours at room temperature, washed, and examined with a Nikon Biophot photomicroscope. The secondary carotenoids formation under nitrogen deficiency was tested according to Kessler and Czygan (1967). After four weeks of growth at a light intensity of 3,000 lux, the colour of the deficient cultures was either orange (secondary carotenoids-positive) or pale greenish-yellow to white (secondary carotenoids-negative).

RESULTS

Protoplast formation in *Chlorella* strains.

In addition to the strains of *C. ellipsoidea* C-87 and *C. saccharophila* C-211 from which protoplasts were formed (Chapter I), 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 II-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 II-1).

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 another. 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. II-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. II-1 a&b); Type 2, the cell wall composed of also two major layers but the outer layer is not trilaminar (Fig. II-1c-g); and Type 3, the cell wall composed of only one microfibrillar layer (Fig. II-1h-l). Obvious intraspecific heterogeneity of cell wall structures existed in C. vulgaris and C. ellipsoidea; there were

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

Strain		Frequency of protoplast formation		
		CMP	CP	DP
C. ellipsoidea	C-87	90	10	0
	C-102	0	0	0
	C-183	0	0	0
C. vulgaris	C-30	0	0	0
	C-133	0	0	0
	C-135	20	10	0
	C-150	1	1	0
	C-169	80	3	30
	C-207	0	0	0
	C-208	0	0	0
	C-209	0	0	0
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 12h, osmotic-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.

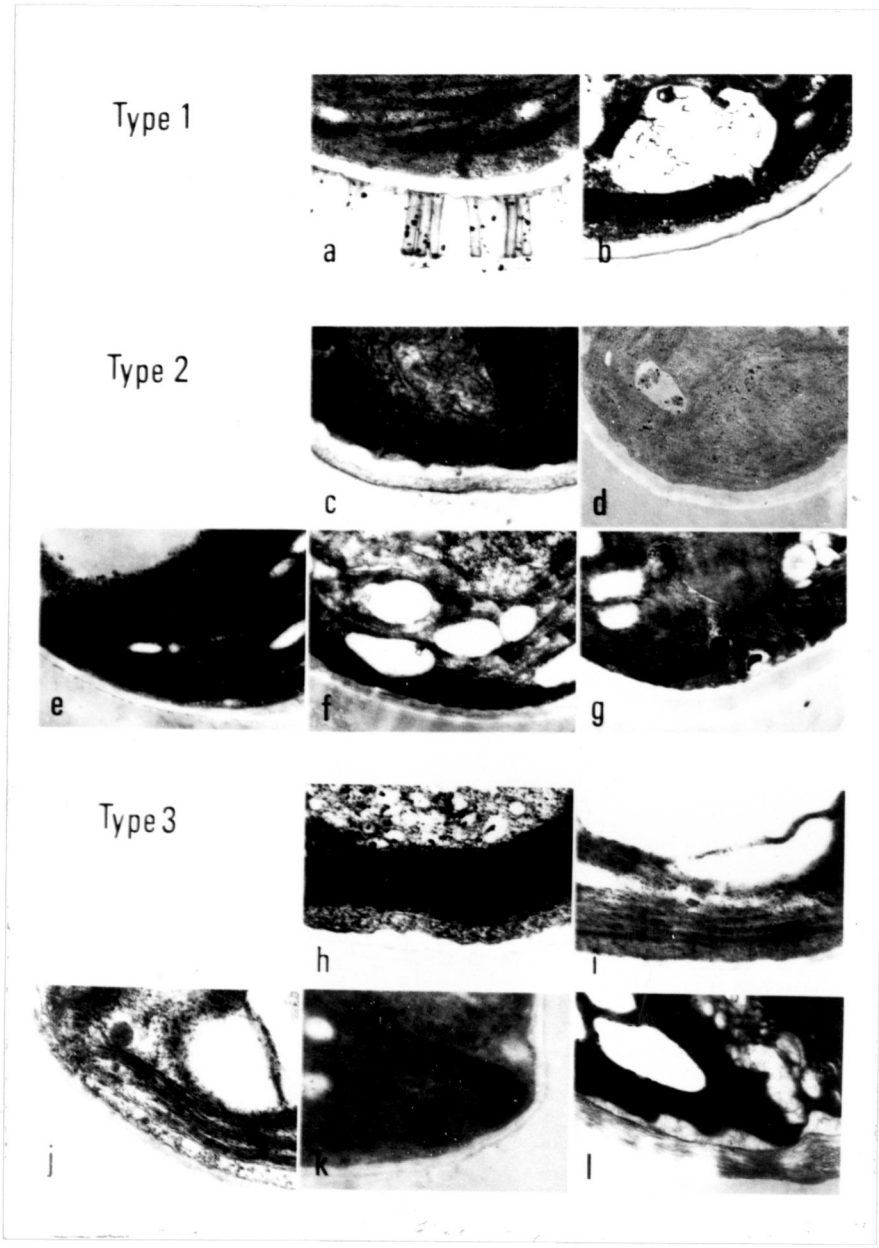


Fig. II-1. Electron micrographs showing thin sections of the cell wall of *Chlorella* strains. (a) *C. ellipsoidea* C-102, (b) *C. vulgaris* C-209, (C) *C. ellipsoidea* C-87, (d) *C. vulgaris* C-169, (e) *C. ellipsoidea* C-83, (f) *C. vulgaris* C-208, (g) *C. saccharophila* C-211, (h) *C. vulgaris* C-30, (i) *C. vulgaris* C-133, (j) *C. vulgaris* C-135, (k) *C. vulgaris* C-150, and (l) *C. vulgaris* C-207. × 30,000.

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. II-1j).

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 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 II 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.

Table II-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-183	++	+	-
	C. vulgaris	C-169	++	+	-
	"	C-208	+	±	-
	C. saccharophila	C-211	++	-	-
3.	*C. vulgaris*	C-30	+	+	-
	"	C-133	+	+	-
	"	C-135	+	+	-
	"	C-150	+	+	-
	"	C-207	+	+	-

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

[b] +, intense red; ±, pink; -, no red colour

DISCUSSION

So far, reports concerning the protoplast formation 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* (Chapter I). 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 (Chapter III). 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 (TableII-1) at somewhat lower frequency. Since the cell wall of C-135 often varied in thickness and consisted of a sparce network (Fig. II-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 strains C-102 possesed a unique cell wall structure, the trilaminar outer layer and the projections on the surface of its cell wall (Fig. II-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 reexamined.

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

SUMMARY

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.

CHAPTER III

Electron Microscopic Studies of *Chlorella ellipsoidea* Protoplast Formation

INTRODUCTION

Causes of the well-known rigidity and indigestibility of *Chlorella* cell wall were shown to be (1) the existence of the sporopollenin layer (Atkinson, *et al.*, 1972), (2) in strains without sporopollenin, the existence of a trilaminar outer layer (Chapter II), and (3) the heterogeneous composition of the cell wall (Chapter II; Soeder, 1962; Takeda and Hirokawa, 1978). The several strains of *Chlorella* which could be induced to form protoplasts in Chapter II were of three closely related species, *C. ellipsoidea*, *C. saccharophila*, and *C. vulgaris*, and possessed a cell wall composed of two layers; an outer thin layer and an inner fibrillar layer.

All strains with a cell wall composed of the two layers, however, could not form protoplasts and for protoplast forming strains, the digestibility of their cell walls by enzymes varied from strain to strain. The relationship between stability and morphology or composition of *Chlorella* cell walls remains yet to be clarified in detail.

In addition, the method of protoplast formation with polysaccharide degrading enzymes should be improved to prevent a deterioration of protoplasts and to save time and troubles.

In this Chapter, I characterize ultrastructural changes during the protoplast formation of *C. ellipsoidea* using transmission and

scanning electron microscopy in order to elucidate the reason why the strain can be induced to form protoplasts, to reveal the relationship between stability and structure of the cell wall, and to improve the method of protoplast formation.

MATERIALS AND METHODS

Experimental cultures. The cells of *Chlorella ellipsoidea* (IAM C-87) were cultured as described in Chapter I.

Preparation of protoplasts. Protoplasts were prepared from freshly harvested cells as described in Chapter I and II.

Electron microscopy. Most procedures were the same as mentioned in Chapter I and II. Portions of the suspension of cells in the enzyme mixture were removed at intervals and centrifuged at 1,000 x g at 4°C. After washing twice with 25 mM phosphate buffer that contained 0.6 M sorbitol/mannitol, cells were fixed with 3 % (w/v) glutaraldehyde in the same buffer for 2 h at room temperature and post-fixed with 1 % OsO_4 in the same buffer for 1 h at 4°C. For scanning electron microscopy, the fixed cells were dehydrated in a graded ethanol series, suspended in acetone, and dried in a HITACHI critical point dryer HCP-1. A small portion of the dried cells was attached to a conductive stub and sputter-coated with gold in a EIKO JB-3 Ion Coater. Samples were examined by a JEM-100CX(JEOL) scanning electron microscope.

For transmission electron microscopy, the fixed cells were embedded in 2 % agar, dehydrated as above, transferred to QY-1 (n-butylglycidyl ether), and immersed in Epon (Millonig and Marinozzi, 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. β-linked polysaccharides in the cell wall were stained with Calcofluor White ST as described in Chapter II. Pectin was stained with Ruthenium Red as in Chapter II.

RESULTS

Initial observations.

The vegitative cells of *C. ellipsoidea* C-87 are ellipsoids of 3-3.5 μm (Fig. III-1a) and are surrounded by a cell wall which consists of two regions (Fig.III-1b): an outer thin layer (10 nm in width) and an inner bulky microfibrillar layer (width from 100 nm to 200 nm). The former layer of cell wall showed higher affinity to the staining with uranyl acetate and lead salts than the latter. No trilaminar outer component of sporopollenin (Atkinson *et al.*, 1972) was observed at any stages of the cell cycle. Whole cells and cell wall fragments showed an intense light blue fluorescence when stained with Calcofluor ST and were positive in the Ruthenium Red stain. Scanning electron micrographs of the vegeta-

tive cells suspended in a hypertonic medium (0.6 % sorbitol/ mannitol, pH 6.0) showed that the surface of cell wall was roughly folded and veinlike furrows extended over the wall (arrows in Fig. III-2a). This was used as a structural marker to distinguish whole cells from protoplasts.

The first step of protoplast formation.

When the cells were incubated for 2-3 h in the protoplast forming medium (ca. 30 % of the cells were osmotically labile, Chapter I), small breaks occurred at or beside a fold on the surface of the cell wall (arrow in Fig. III-3a). Thin sections of the cells at this stage showed that the inner region of the cell wall had almost disappeared and that the outer region had been irregularly released apart from the plasma membrane (Fig. III-3b). Staining with Calcofluor, the cells at this stage showed only very faint or no blue fluorescence (Fig. III-4).

The second step of protoplast formation.

Figures III-5a and b show scanning electron micrographs of cells incubated for 4-5 h (ca. 50 % of the cells were osmotically labile). Most vegitative cells had large breaks on their outer layer of the cell wall and through them, a rugged surface of the plasma membrane was exposed (arrows in Fig. III-5a and b). Figure III-5c shows a thin section of the cell at this stage; the outer region of the cell wall is considerably degraded (arrows).

The third step of protoplast formation.

After this stage the remaining portions of the cell wall occasionally came off and almost spherical protoplasts appeared into the medium (Fig. III-6a); the empty wall structure was beside the protoplast (arrows in Fig. III-6a and b). After 6-8 h incubation, most of the cell population have converted to protoplasts (Fig. III-7). Figure III-8a shows a scanning electron micrograph of a typical protoplast which is spherical, 4.5 μm in diameter with a somewhat rugged surface, and without any fragments of the cell wall. The protoplastic nature was confirmed by a thin section shown in Fig. III-8b; the plasma membrane was wavy and without any fragments of the cell wall.

Regeneration of the cell wall and growth of the protoplasts.

Some of the protoplasts suspended in the culture medium containing 20 % sucrose could after 3 to 4 days incubation at 25°C in the light (3,000 lux) regenerate the cell wall which was stained with Calcofluor (Fig. III-9). When the protoplasts were embedded in 0.6 % soft agar-culture medium containing 20 % sucrose and 0.1 % yeast ex. (Difco), up to 30 % of the protoplasts could grow and form colonies on the plates after 2 week incubation at 25°C in the light.

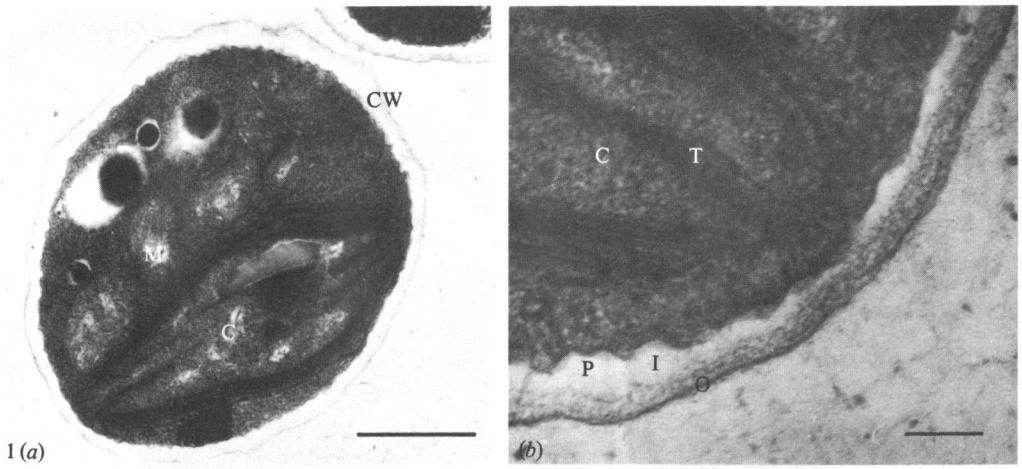


Fig. III-1. Electron micrographs showing an intact vegetative cell of C. ellipsoidea.

(a) Thin section of a whole cell. C. chloroplast; CW, cell wall; M. mitochondrion. (b) Thin section of the cell wall. C, chloroplast; I, inner region of cell wall; O, outer region of cell wall; P, plasma membrane; T, thylakoid. Bar marker represents 1 μm in (a) and 0.2 μm in (b).

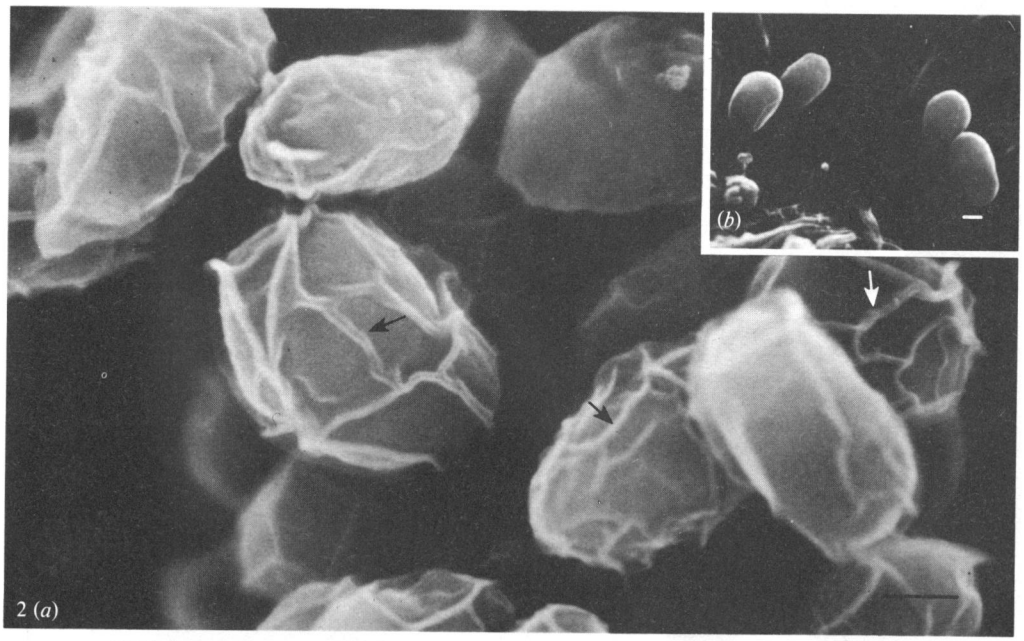


Fig. III-2. Scanning electron micrograph of C. ellipsoidea cells.

(a) Cells suspended in 0.6 M sorbitol/mannitol (1:1) at pH 6.0. Arrows indicate furrows on the surface of the cell wall. (b) Cells in the growth medium. The surface of the cell wall is smooth. Bar marker represents 1 μm.

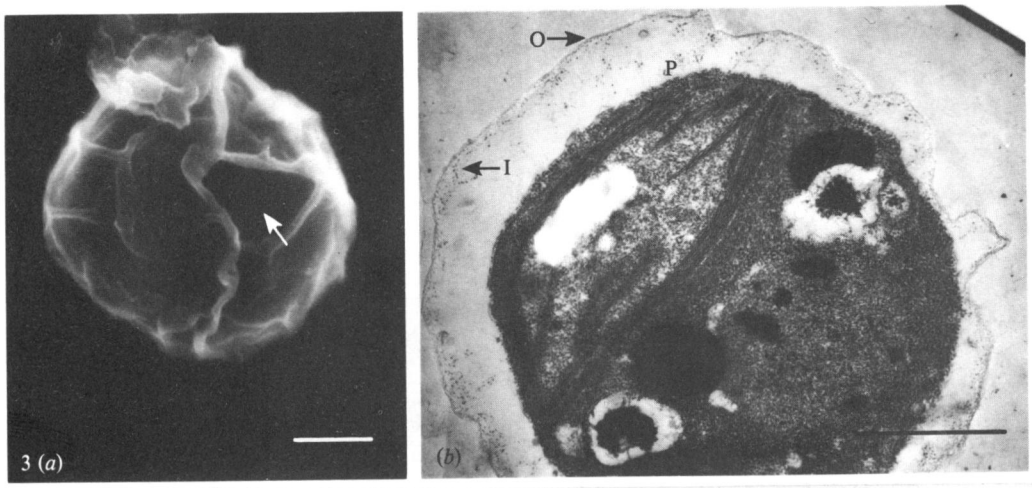


Fig. III-3. Electron micrographs showing cells of C. ellipsoidea at an intermediate stage of the protoplast formation. (a) Scanning electron micrograph showing a cell incubated for 3 h in the protoplast induction medium. The cell surface is roughly folded. A small split occurs at a hold point (arrow). (b) Thin section of a cell incubated for 3 h. The outer region (O) of cell wall is released from the plasma membrane (p). The inner region (I) is considerably degraded. Bar marker represents 1 μm.

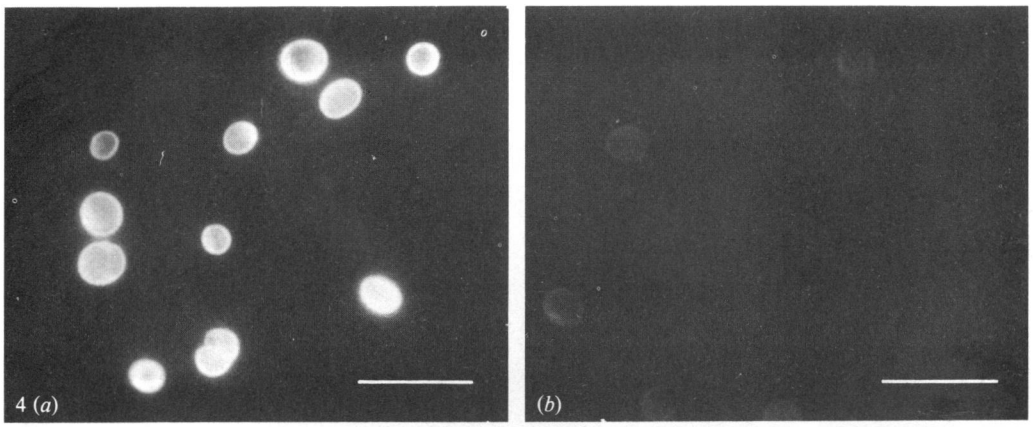


Fig. III-4. Cell wall stained with Calcofluor white ST. (a) Whole cells at an exponentially growing phase. Cell wall was strongly stained and bright. (b) Cells treated with the enzyme mixture for 3 h. Fluorescence around the cells was very faint. Bar marker represents 10 μm.

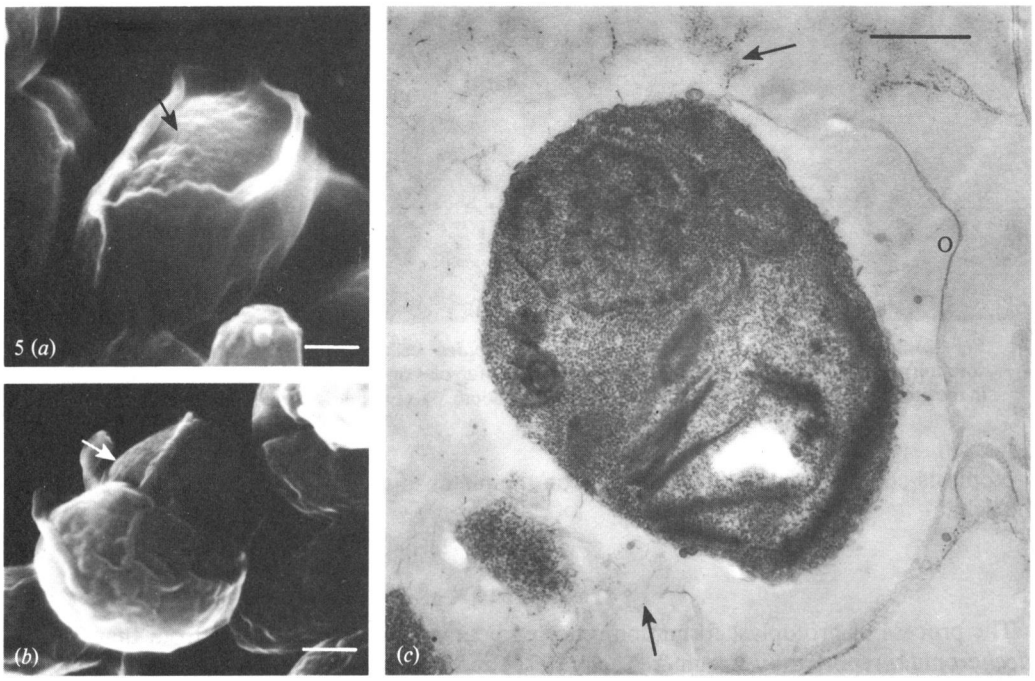


Fig. III-5. Electron micrographs showing cells of C. ellipsoidea in the process of the protoplast formation. (a,b) Scanning electron micrographs of cells incubated for 5 h. Most of the cells have large breaks on their cell wall. A rugged surface of plasma membrane is exposed through the breaks on the cell wall (arrows). (c) Thin section of a cell incubated for 5 h. The outer region of cell wall (O) is degraded at some sites (arrows). Bar marker represents 1 μm.

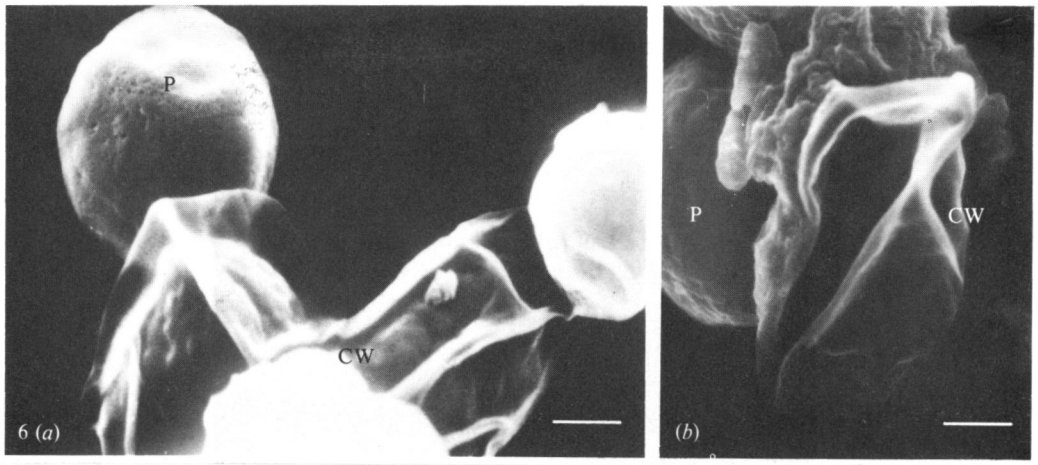
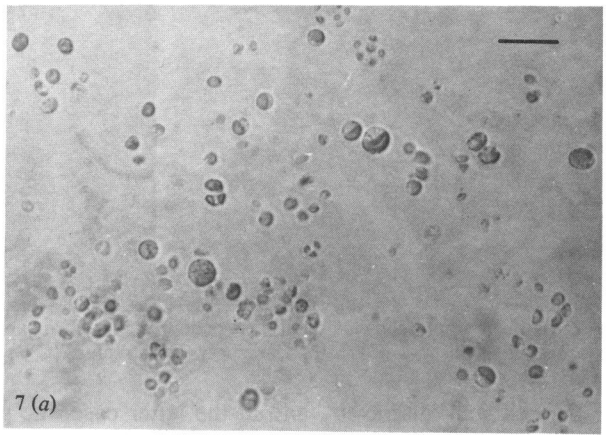


Fig. III-6. Scanning electron micrographs showing the last stage of the protoplast formation. (a) A protoplast (P) came out from a degrading cell wall (CW). (b) The empty wall structure (CW) is remaining beside the protoplast. Bar marker represents 1 μm.



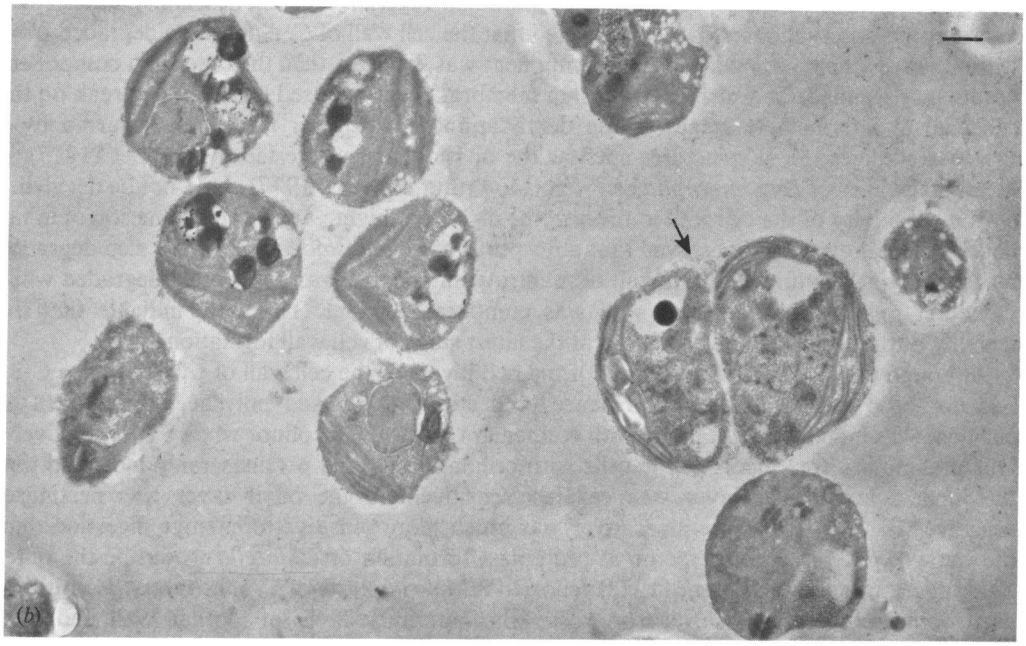
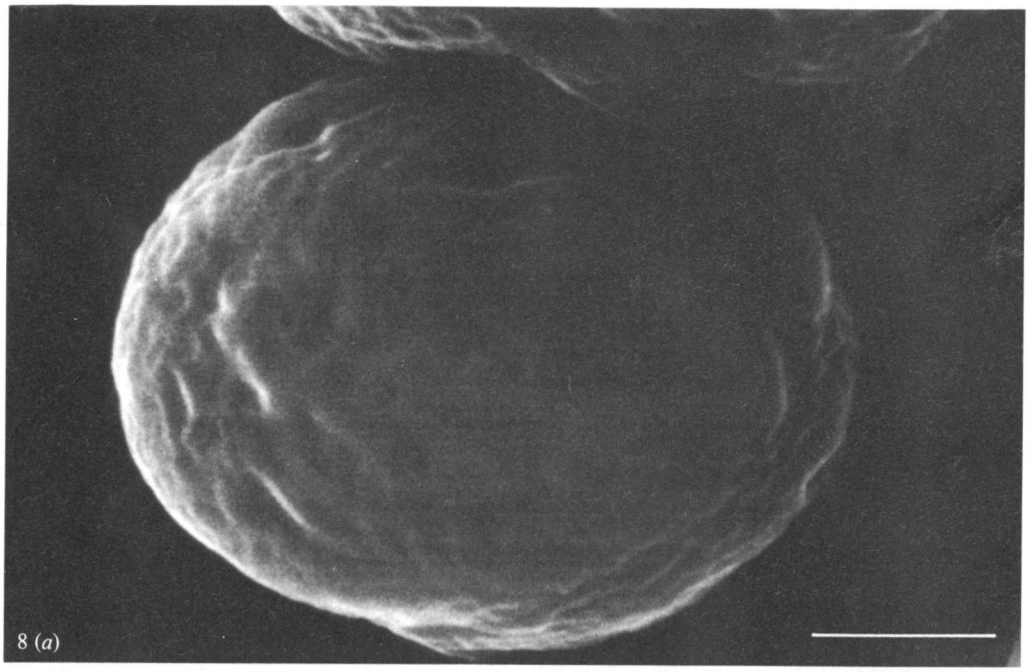


Fig. III-7. Cells of C. ellipsoidea after the incubation for 8 h. (a) Optical micrograph; most cells have converted to spherical shape. There are a few large swallen cells. (b) Thin section of the cells. Most cells are without cell wall structures; some cells still retain the degrading outer layer of cell wall (arrow). Bar marker represents 10 μm (a) and 1 μm (b).



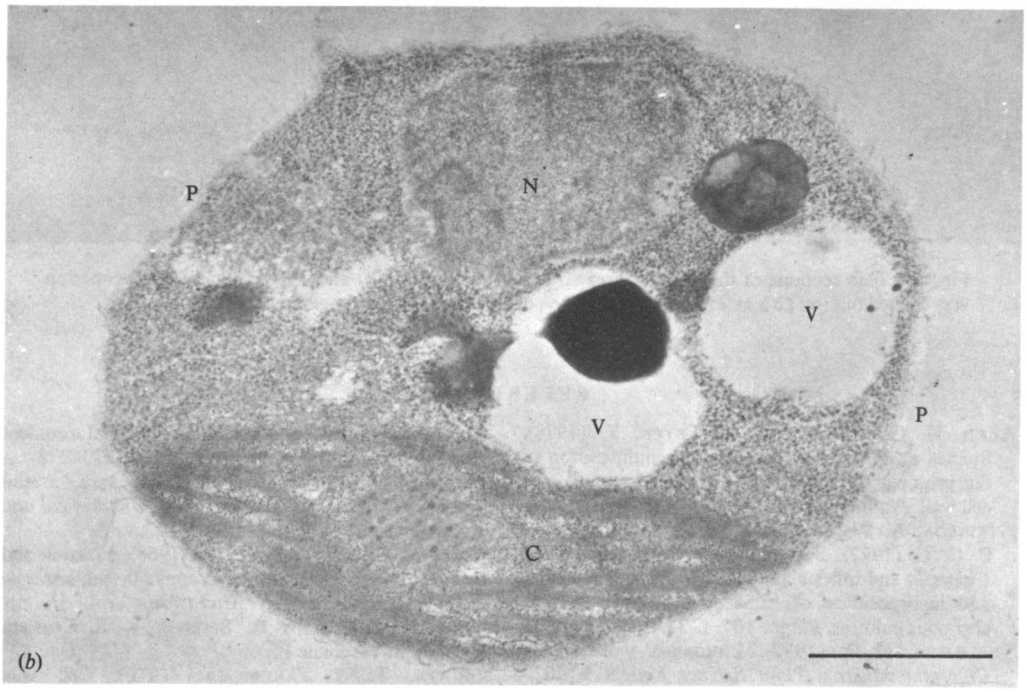


Fig. III-8. Electron micrographs showing the protoplast of C. ellipsoidea after the incubation for 8 h. (a) Scanning electron micrograph. The surface of plasma membrane finely rugged. (b) Thin section of the protoplast. Plasma membrane (p) is wavy. C, chloroplast; N, nucleus; P, plasma membrane; V, vacuole. Bar marker represents 1 μm.

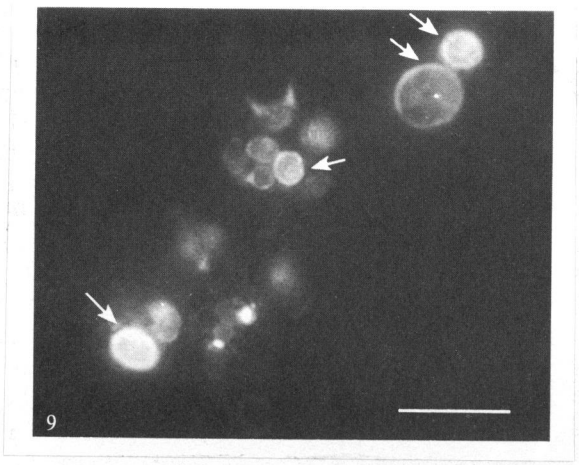


Fig. III-9. Staining with Calcofluor white ST of protoplasts incubated in a regeneration medium. After 3 days incubation at 25°C in the light (3,000 lux), some cells in the protoplast aggregates were re-stained and brightened (arrows). Bar marker represents 10 μm.

DISCUSSION

The process of protoplast formation reported here is somewhat different from that for C. saccharophila reported by Braun and Aach (1975) and that for C. vulgaris by Berliner (1977). In the former case, the cell wall "dissolved", and in the latter case, the protoplasts extruded through breaks in the cell wall, probably through vegetative division scars. Our electron microscopic observations, however, revealed that the cell wall of C. ellipsoidea degraded from within: firstly, the inner microfibrillar component was digested; then, the outer thin component gradually degraded. The surface of plasma membrane was exposed through the break on the cell wall at intermediate stages of the degradation, but whether the break occurred by a site-specific digestion at structures such as the division scar suggested by Berliner (1977) or the damage lines of Enteromorpha cell wall (McArthur and Moss, 1977) could not be decided.

The sensitivity of the cell wall to the enzymatic digestion might vary during some stages in the developmental cycle. In the course of the present study, it was also learned that autospores of C-87 were also degraded by the enzyme mixture; the cell wall of maturing autospores were completely degraded while the outer region of mother cell wall was comparably intact. It may indicate that the stability of the outer region is aquired at the latest stage of cell wall formation.

Though chemical composition of the both layers of the cell wall of C. ellipsoidea C-87 has not been fully studied, at least pectin and β-linked polysaccharides such as cellulose were detected by the stain method with Ruthenium Red and Calcofluor White ST, respectively. Our preliminary treatments of the cells with cellulase only and pectinase only indicated that the inner microfibrillar layer was cellulase-sensitive and the outer layer was pectinase-sensitive (Fig.III-10). Since the inner layer was much more sensitive to enzymatic digestion than the outer layer, the limiting factor of protoplast formation of Chlorella would be the outer rigid layer; in fact, Aach et al., (1978) reported that protoplasts of C. saccharophila could be easily obtained by cellulase digestion after the outermost layer of the cell wall had been scratched with sea sand. The differences in digestibility of Chlorella cell walls observed among protoplast forming strains may be due to a species-specific structure or composition of the outer layer of cell wall.

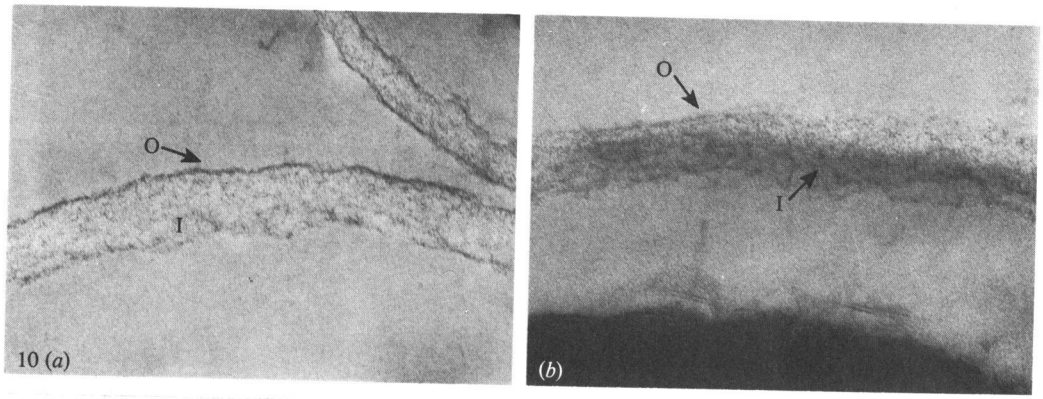


Fig. III-10. Thin sections of the cell wall treated with 4% cellulase ONOZUKA (2) and 1% pectinase (b). Incubation was carried out for 12 h at 25°C. I, inner region of cell wall; O, outer region of cell wall.

SUMMARY

The intermediate stages of protoplast formation of *Chlorella ellipsoidea* were studied by transmission and scanning electron microscopy. Within the first few hours of treatment with a polysaccharide degrading enzyme mixture, the microfibrillar inner component of the cell wall was almost completely digested and the remaining outermost component was released irregularly from the plasma membrane. As incubation proceeded, a crevice which occurred at a fold of the outer wavy layer gradually extended through the layer; then, the surface of the plasma membrane was exposed from the crevice. The gradually degrading outer layer peeled off and an almost spherical protoplast with fine furrows on its surface was released.

Degradation of the outermost thin layer of cell wall, probably composed of pectin, limits the rate of protoplast formation in *C. ellipsoidea*.

CHAPTER IV

Isolation and Characterization of Chloroplast DNA from *Chlorella ellipsoidea*

INTRODUCTION

The green alga represented by the genus Chlorella includes species that considerably differ in biochemical and physiological characteristics (Kessler, 1976). The GC content of the DNAs from different species ranges from 43 to 79 % (Hellmann and Kessler, 1974). Hybridization of DNAs from different species of Chlorella to one another indicate that there are several distinctively different groups of species within this genus (Kerfin and Kessler, 1978). In contrast, studies on chloroplast DNAs (cp DNAs) from different strains of Chlorella by means of thermal denaturation and renaturation kinetics showed that this genome was quite homogeneous within this genus (Dalmon and Bayen, 1975) and similar to those of other algae such as Chlamydomonas and Euglena (Bayen and Rode, 1973). Recently, the cp DNAs of Chlamydomonas reinhardii (Rochaix, 1978; Rochaix, 1981) and of Euglena gracilis bacillaris (El-Gewely et al., 1981) have been characterized. Both cp DNAs were physically mapped with restriction endonucleases and some specific genes (e.g. rRNAs, etc) were located on these maps. The cp DNA of Chlamydomonas was also shown to be methylated during certain stages of the life cycle of this alga (Royer and Sager, 1979). The cp DNAs of these two algae are physically organized quite differently. This raises the question as to the organization of other algal cp DNA such as those from the genus Chlorella.

Although the existence of a rigid and indigestible cell wall have prevented an isolation of intact cellular components from Chlorella cells, I established a method to digest the cell wall and to isolate intact Chlorella protoplasts in Chapter I and II. In Chapter I, I also suggested the possibility of isolation of intact chloroplasts and cp DNA from Chlorella protoplasts.

This Chapter includes the isolation of an intact circular cp DNA of C. ellipsoidea C-87, the measurement of its buoyant density (GC content) and contour length (molecular weight), and some restriction endonuclease fragment patterns.

This is the first to report an isolation of intact cp DNA from Chlorella. It was not until a successful isolation of protoplasts from Chlorella that this study became possible.

MATERIALS AND METHODS

Plant material. The cells of Chlorella ellipsoidea (IAM C-87) were cultured as described in Chapter I.

Preparation of protoplasts and chloroplasts. Protoplasts were obtained as in Chapter I and II. For the isolation of chloroplasts, the prepared protoplasts were washed and resuspended in the homogenization medium A (Kolodner and Tewari, 1975) with a modification (0.3 M mannitol was replaced by 0.3 M sorbitol). The protoplast suspension (10^9 cells / ml) was homogenized at 0°C by a glass

homogenizer with a motor-driven Teflon pestle. The homogenate was centrifuged at 1,000 rpm for 5 min to remove unbroken cells and autospores. The supernatant (about 5 ml) was then layered onto a discontinuous density gradient containing 1.0, 1.5, 2.0, and 2.5 M sucrose (Sager and Ishida, 1963). The gradients were centrifuged at 4°C in an RPS 25 rotor at 25,000 rpm for 90 min in a Hitachi 65P ultracentrifuge. The green bands corresponding to chloroplasts (Sager and Ishida, 1963) were harvested. The uppermost band was found to be composed mainly of broken chloroplasts and the second one of intact chloroplasts.

Isolation of cp DNA. Chloroplast DNA for electron microscopic observations was prepared according to Kolodner and Tewari (1975). Chloroplast DNA for restriction endonuclease analyses was prepared as described by Kolodner and Tewari (1972) and purified twice by ultracentrifugation in a neutral CsCl gradient (ρ=1.700 g/cm^3). Analytical CsCl density equilibrium ultracentrifugation in neutral CsCl solution was performed at 40,000 rpm for 30 h at 25°C in a Hitachi analytical ultracentrifuge Model 282, with phage SP01 DNA (ρ=1.741 g/cm^3), *Escherichia coli* DNA (ρ=1.710 g/cm^3), and *Bacillus subtilis* DNA (ρ=1.703 g/cm^3) as density markers.

Restriction endonuclease analyses. Restriction endonucleases *Bam*HI, *Bcl*I, *Bgl*I, *Hae*II, *Sst*I, *Sst*II, and *Xho*I were purchased from Bethesda Research Labs (USA); *Bgl*II, *Eco*RI, *Pst*I, and *Sma*I from Takara Shuzo Co. Ltd. (Japan); *Hpa*II from Boheringer Mannheim (FRG); and *Msp*I and *Pvu*I from New England Biolabs (USA).

XmaI was purified according to Endow and Roberts (1977). Chlorella cp DNA was incubated at 37°C for 1 h with a sufficient amount of restriction endonucleases to effect complete digestion in the buffers recommended by the suppliers. Double digestion with KpnI and SstI or XhoI was carried out in the buffer for KpnI. The buffer for SstI was used for the double digestion with SstI and XhoI. Agarose gel electrophoresis of DNA fragments was carried out according to Rochaix (1978). EcoRI and HindIII fragments of λDNA were used as molecular weight markers.

Electron microscopy of Chlorella cp DNA. Chloroplast DNA in 10 mM Tris-HCl (pH 8.5) containing 1 mM EDTA, 0.03 % cytochrome C and 50 % formamide was spread onto the hypophase consisting of 1 mM Tris-HCl (pH 8.5), 0.1 mM EDTA, and 20 % formamide according to Davis et al. (1971). The spreads were examined in a JEM-100B (JEOL) electron microscope after rotary shadowing with Pt-C. Contour length of circular cp DNA molecules was determined by comparison with plasmid pBR322 DNA (Sutchiffe, 1979).

RESULTS

Isolation of cp DNA.

DNA prepared from protoplasts of C. ellipsoidea showed two UV absorbing bands in neutral CsCl density equilibrium gradient (Fig. IV-1a); the minor band at a density less than 1.700 g/cm^3

accounted for about 8 % of total DNA. When DNA was prepared from isolated chloroplasts, the band at the lower density accounted for more than 85 % of total DNA (Fig. IV-1b) suggesting that it be identified as cp DNA band. Although the band of cp DNA was greatly enriched (about ten times), the DNA of the higher density could not be completely eliminated even by the treatment of chloroplast preparations with DNase (Kolodner and Tewari, 1975). For restriction endonuclease analyses, the cp DNA fractions were repurified by ultracentrifugation in CsCl gradient.

Characterization of cp DNA.

The buoyant densities of the cp DNA and nuclear DNA in CsCl equilibrium density gradients were found to be 1.695 ± 0.002 g cm^{-3} and 1.717 ± 0.002 g cm^{-3}, respectively (Fig. IV-2). The densi of cp DNA corresponds to GC content of 35.7 % (in molar percent) in the DNA (Schildkraut *et al.*, 1962) which is comparable to that of cp DNAs from higher plants and several algae (Herrmann and Possingham, 1980). Electron micrographs of the cp DNA isolated by CsCl-ethidium bromide ultracentrifugation (Kolodner and Tewari, 1975) revealed circular molecules and linear fragments (Fig. IV-3a) most circles were in the open configuration and some were supercoiled. In Fig. IV-3b, the contour lengths of several circular cp DNA molecules from *C. ellipsoidea* are given in pBR322 units (4362 base pairs) (Sutchiffe, 1979). From these determinations the size population of the cp DNA circles appeared to be uniform.

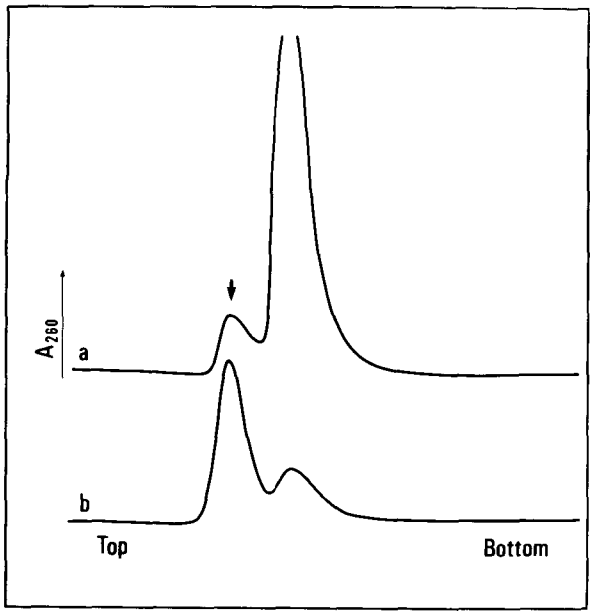


Fig. IV-1. Absorbance profile of DNAs from C. ellipsoidea, separated by preparative CsCl gradient centrifugation. (a) DNAs from protoplasts and (b) from isolated chloroplasts. Chloroplast DNA was considerably enriched (arrow).

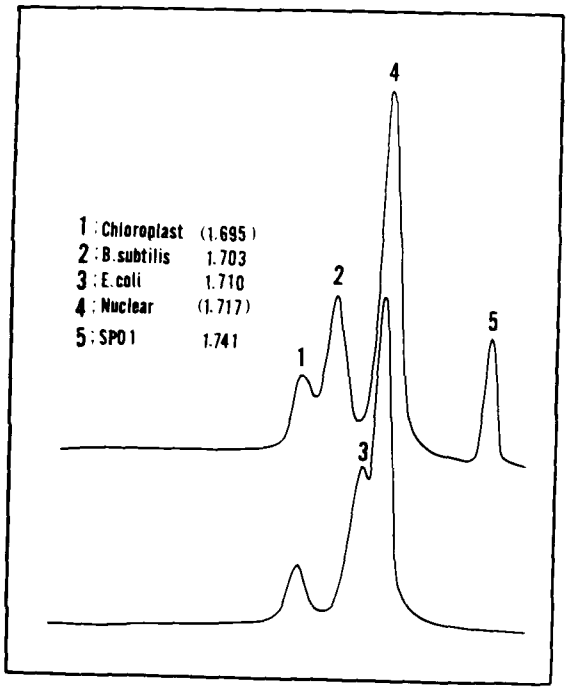
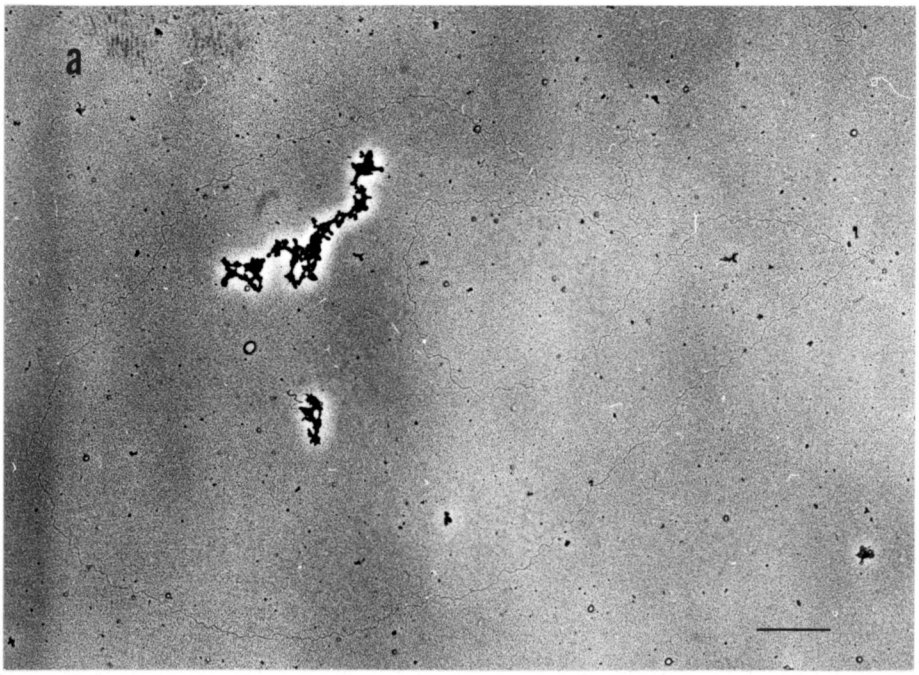


Fig. IV-2. Photoelectric scans from analytical ultracentrifugations of C. ellipsoidea DNAs. The buoyant densities of cp DNA and nuclear DNA were determined as 1.695 and 1.717 (g cm^{-3}), respectively. Density markers used were as follows: DNA of B. subtilis (1.703 g cm^{-3}), of E. coli (1.710 g cm^{-3}), and of phage SPO1 (1.741 g cm^{-3}).



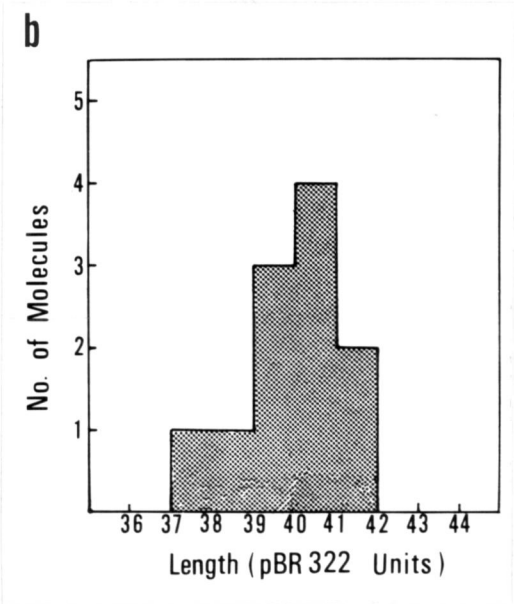


Fig. IV-3. Electron micrograph of an open circular cp DNA molecule of *C. ellipsoidea* (a). Bar marker represents 1 μm. (b) Length frequency distribution of the cp DNA. Size was presented in pBR322 units (4,362bp).

The mean length was calculated as 40 pBR322 units which corresponds to 26 μm, 175 kilobase pairs, or 115 × 10^6 dalton. The size was also determined by restriction endonuclease analyses where the cp DNA was digested with KpnI, SstI, XhoI, KpnI + SstI, KpnI + XhoI, and SstI + XhoI (Fig. IV-4). A list of DNA fragments including their sizes and stoichiometries is given in Table IV-1. The largest size (about 170 Kbp) obtained with SstI agrees with the size of cp DNA determined by electron microscopy.

Restriction endonuclease patterns of cp DNA.

Since the intramolecular heterogeneity of cp DNA from *Chlorella* strains was known (Bayen and Rode, 1973; Dalmon and Bayen, 1975), the fragment patterns of cp DNA from *C. ellipsoidea* produced by the digestion with several restriction endonucleases were studied. Figure IV-5(a & b) shows the restriction patterns of the cp DNA digested with EcoRI, BglI, BglII, HaeII, KpnI, PstI, SstI, BamHI, and XhoI. From the size and the base composition of the DNA, and from the specificity of each restriction endonuclease, the number of cleavage sites per cp DNA is expected as follows: 57.8 for EcoRI and BglII; 18.2 for BamHI, KpnI, PstI, SalI, SstI, and XhoI; 24.0 for HaeII; 5.8 for BglI. In most cases, the observed number of sites agree with the predicted values: 23 for EcoRI; 11, BglI; 22, BglII; 30, HaeII; 14, KpnI; 19, PstI; 20, SstI; 18, XhoI. BamHI and SalI, however, cut the cp DNA significantly less.

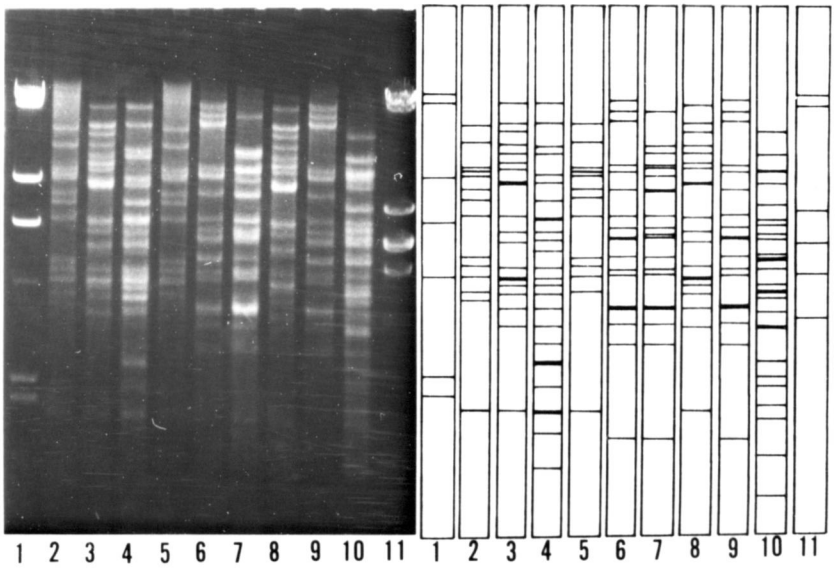


Fig. IV-4. Agarose gel electrophoresis for size determination of cp DNA from C. ellipsoidea after cleavage with several restriction endonucleases: (1) λ DNA digested with Hind III, (2) Kpn I, (3) Sst I, (4) Kpn I + Sst I, (5) Kpn I, (6) Xho I, (7) Kpn I + Xho I, (8) Sst I, (9) Xho I, and (10) Sst I + Xho I cleavage pattern of cp DNA, and (11) λ DNA digested with EcoRI (all on the same 0.5 % agarose gel).

Table IV-1. Sizes of DNA fragments resulting from digestion with several restriction endonucleases of cp DNA from C. ellipsoidea (in Base Pairs)[a]

Restriction Enzymes	KpnI	SstI	XhoI	KpnI/SstI	KpnI/XhoI	SstI/XhoI
Fragment No.						
1	>30[b]	23.5	21.7	23.5	20.0	15.0
2	16.3	17.0	20.0	17.0	12.6	12.1
3	13.2	15.0	17.5	12.7	11.8	10.1(2)
4	10.5	12.9	10.7	12.1	10.8	9.00
5	9.61	12.1	9.76	9.40	10.7	7.65
6	9.40	10.8	8.49	9.00	9.40	6.80
7	8.49	10.4	7.01	8.08	8.49(2)	6.55
8	8.08	9.15(2)[c]	6.35	6.82(2)	6.95	6.15
9	6.95	6.80	6.00(2)	6.25	6.35	5.95
10	5.25	6.25	5.27	5.78	6.15	5.30
11	4.87	5.78	4.85	5.50	6.00	5.25(2)
12	4.52	4.80	4.65	4.95	5.27	4.85
13	4.08	4.40(2)	3.72(2)	4.40	4.80	4.40
14	3.85	4.25	3.30	4.25	4.65	4.20(2)
15	1.80	4.00	2.85	4.00	3.72(2)	3.95
16		3.64	1.95	3.64	3.30	3.60
17		3.22		3.22	2.85	3.26(2)
18		1.80		2.86	1.95	3.55
19				2.45(2)		2.30
20				2.16		2.12
21				1.77(2)		1.87
22				1.53		1.69
23				1.19		1.29
24						1.19
Sum	>136.9	169.34	143.82	163.59	147.99	150.93

[a] Determined from the electrophoretic mobility in 0.5 % agarose gel.

[b] Large smear bands; the sizes could not be determined.

[c] The numbers in parentheses indicate a probable stoichiometry of 2.

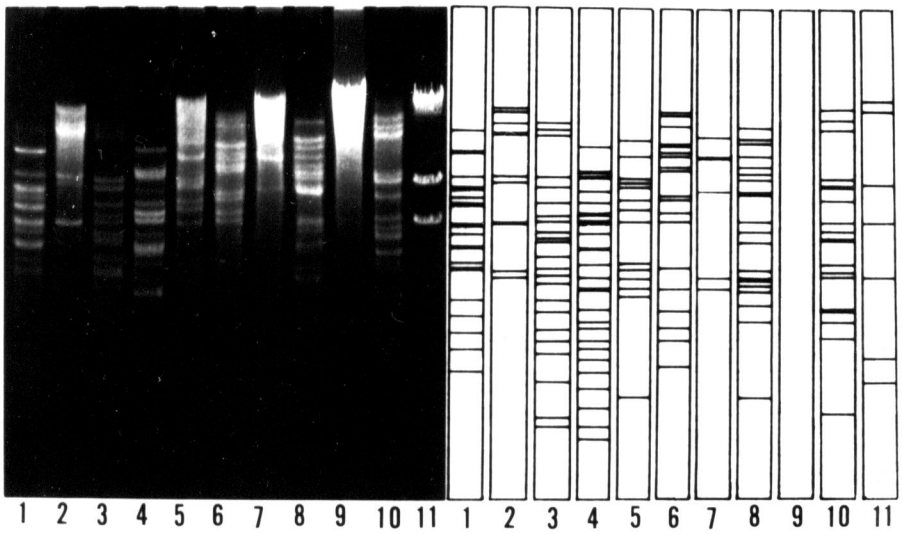


Fig. IV-5. Agarose gel electrophoresis of cp DNA after cleavage with various restriction endonucleases: (1) EcoRI, (2) Bgl I, (3) Bgl II, (4) Hae II, (5) Kpn I, (6) Pst I, (7) Sal I, (9) Sst I, (10) BamH I, and (11) Xho I fragments of cp DNA and (12) λ DNA digested with Hind III (0.5 % agarose gel).

Recently, DNA modification in eukaryotes, such as the methylation of C residues, has been demonstrated and discussed with respect to its significance (Ehrlich and Wang, 1981). For the green alga Chlamydomonas reinhardii, the methylation of cp DNA is known to play an important role in the maternal inheritance of chloroplast (Sager et al., 1981). Therefore, the cp DNA of Chlorella was tested for the methylation of C residues. Figure IV-6 shows a comparison of the restriction fragment patterns with SmaI, XmaI, HpaII, and MspI, which were used to probe for methylation of DNA (Royer and Sager, 1979; Youssoufian and Mulder, 1981). If the methylation at the internal CG sites of cp DNA occurs, SmaI which recognizes the sequence of CCCGGG and HpaII (CCGG) do not cleave the DNA in contradistinction to their isoschizomers XmaI and MspI, respectively. Since most band patterns were the same between the iso scizomers (Fig. IV-6), however, the methylation at the CG sites were very rare if they occurred. On the other hand, the isoschizomers gave different band patterns, when the nuclear DNAs of C. ellipsoidea was digested. This indicated a low level of methylation at the CG sites.

DISCUSSION

The cp DNA from C. ellipsoidea is a large circular molecule with a buoyant density of 1.695 g cm^{-3} (36 % GC). In this respect,

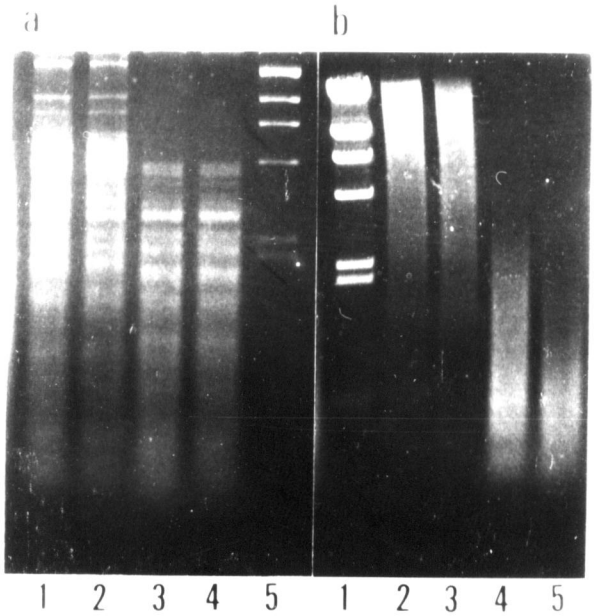


Fig. IV-6. Agarose gel electrophoresis of cp DNA (a) and nuclear DNA (b) after cleavage with restriction enzymes Sma I, Xma I, Hpa II, and Msp I. (a) Cp DNA digested with Sma I (1), Xma (2), Hpa II (3), and Msp I (4). λ DNA digested with Hind III was used as a size marker (5). (b) Nuclear DNA digested with Sma I (2), Xma I (3), Hpa II (4), and Msp I (5). λ DNA-Hind III marker was in (1). Electrophoreses were carried out on 0.7 % agarose gel.

it is similar to those reported for several higher plant and algae (Herrmann and Possingham, 1980). However, the size of the cp DNA of C. ellipsoidea (175 kilobase pairs, 115 × 10^6 dalton) is considerably larger than those of higher plant whose molecular weights are reportedly at the most 100 × 10^6 dalton (Herrmann and Possingham, 1980); one exception is the cp DNA of a duckweed Spirodela oligorrhiza which was reported to have a size of 115 to 120 × 10^6 dalton (Van Ee et al., 1980). In algae, Euglena gracilis (Euglenophyceae) and Vaucheria sessilis (Xanthophyceae) possess a very small size of cp DNA (Herrmann and Possingham, 1980). Chlamydomonas reinhardii as well as Chlorella ellipsoidea (Chlorophyceae) seems to have a larger cp DNAs: the size of C. reinhardii cp DNA, 126 × 10^6 daltons (Rochaix, 1978). The size variation of cp DNAs from various origins may have some evolutionary significance. In this connection, DNA from cyanelles of Cyanophora paradoxa has a size of 110 × 10^6 dalton, though its buoyant density is very high (1.716) (Jaynes et al., 1981).

Restriction endonuclease analyses of the Chlorella cp DNA revealed that the sites for some enzymes such as BamHI (GGATCC) and SalI (GTCGAC) were significantly fewer than expected (Fig. IV-5) PvuI (CGATCG) and BclI (TGATCA) were also ineffective to the cp DNA (data not shown). This may be due to some modifications at these sites or to a nonrandom base arrangement of the cp DNA. The methylation of the C residue of cp DNA such as the one reported for Chlamydomonas reinhardii (Sager et al., 1980) might be the case

for C. ellipsoidea, though the methylation at the internal CG sites of the sequence CCGG or CCCGGG was absent in the Chlorella cp DNA. A methylation at other sites of the DNA might have occurred. Indeed, some methylation enzymes were isolated and characterized in C. reinhardii (Sano and Sager, 1980; Sano et al., 1981), one of which existed in vegetative cells and was specific to the base sequence of TCG, TCC and TCA.

The cp DNA used in this study was isolated from nonsynchronously and photosynthetically growing cells of C. ellipsoidea; it is interesting to compare the present results with those of cp DNA from cells at various growth phases in a synchronous culture or from cells growing under different conditions (heterotrophically or mixtotrophically).

Basing on the restriction endonuclease patterns obtained in this work, a physical map of the cp DNA from C. ellipsoidea is now to be constructed. Some specific genes such as for rRNAs or for RuBPCase will be located on the map.

SUMMARY

A circular DNA molecule was isolated from chloroplasts of *Chlorella ellipsoidea*. The DNA had a buoyant density of 1.695 g cm^{-3} (36 % GC) and a contour length of 56 μm (175 kilobase pairs). The restriction endonuclease analysis gave the same size. Agarose gel electrophoretic patterns of cp DNA digested by several restriction endonucleases were also presented. The digestion by the restriction enzymes, *HpaII*, *MspI*, *SmaI*, and *XmaI* revealed no appreciable methylation at CG sites in cp DNA.

CHAPTER V

Characterization of Inverted Repeat Sequences and Ribosomal RNA Genes of Chloroplast DNA from *Chlorella ellipsoidea*

INTRODUCTION

The chloroplast DNAs (cp DNAs) from a wide range of higher plants (angiosperms) share many common properties: circular conformation, size of ca. 150 kbp, low GC content, physical arrangement, and map position of several genes (Harrmann and Possingham, 1980; Bogorad, 1981; Wallace, 1982). A characteristic structural feature of those cp DNAs is the existence of large inverted repeat sequences of 22∿25 kbp in size, part of which codes for chloroplastic ribosomal RNA genes. The inverted repeat sequences are suggested to play a direct role in the conservation of some base arrangements of cp DNA sequences (Palmer and Thompson, 1982).

As for the cp DNAs from non-angiosperms, much less has been so far studied. Recently, Palmer and Stein (1982) characterized a cp DNA from a fern Osmunda whose properties are remarkably similar to those of cp DNAs from higher plants, although the inverted repeats are somewhat smaller in the fern cp DNA (8∿13 kbp). Chloroplast DNAs from other two ferns and one liverwort have been reported to be relatively homogeneous in size (130∿150 kbp) and in some properties (Wallace, 1982).

On the other hand, a significantly greater size heterogeneity is observed among cp DNAs from several green algae: from ca. 85 kbp for Codium fragile (Hedberg et al., 1981) to 1670 ∿ 2270 kbp for Acetabularia (Padmanabham and Green, 1978). Considerable variations in the physical arrangement of cp DNAs have also been

known among algal species. Only two algal cp DNAs have so far been well characterized: those of Chlamydomonas reinhardii (Rochaix, 1978) and Euglena gracilis bacillaris (Jenni and Stutz, 1978; Rawson et al., 1978; Gray and Hallick, 1978). These two genomes are physically organized quite differently not only from each other but also from those of higher plants. The cp DNA from Chlamydomonas reinhardii is a large circular molecule of 190 kbp containing a set of inverted repeats of 19 kbp on which rRNAs (23S, 16S, 7S, 5S, and 3S) are located. On the other hand, the cp DNA from Euglena gracilis bacillaris (134 kbp) possesses three tandemly repeated rRNA operons.

Several interesting features were also known for Chlamydomonas cp DNA: (1) the occurrence of methylated nucleotides in the DNA (Royer and Sager, 1979) and (2) the existence of intervening sequences in the 23S rRNA gene (Rochaix, 1981).

Since the heterogeneity of the cp DNA structure observed in several algae might reflect the degree of evolutional divergence in the algal world it would be great interest to investigate the physical properties and the molecular arrangement of cp DNA's from such a familiar and ubiquitous alga as Chlorella.

Recently, Yamada (1982) isolated and characterized the cp DNA from Chlorella ellipsoidea. It was a large circular molecule (ca. 175 kbp) with a buoyant density of 1.695 g/cm^3 (36% GC); the physical properties were similar to those of Chlamydomonas reinhardii (Herrmann and Possingham, 1980). The present study

gives further characterization of the cp DNA using electron microscopy and restriction enzyme analysis. It also demonstrates that certain structural features of the cp DNA such as the arrangements of inverted repeats and of rRNA genes are more similar to those of higher plants than of other algae, Chlamydomonas and Euglena.

MATERIALS AND METHODS

Preparation of intact cp DNA. Chlorella ellipsoidea Gerneck (IAM C-87) was obtained from the algal culture collection of the Institute of Applied Microbiology, University of Tokyo. From freshly harvested cells, protoplasts were prepared as described elsewhere (Yamada and Sakaguchi, 1981; Yamada and Sakaguchi, 1982). Chloroplasts and cp DNA were isolated and purified by ultracentrifugation after homogenization of the protoplasts (Yamada, 1982).

Self-annealing of inverted repeats. The purified cp DNA was dissolved in a spreading mixture (2μg/ml; 30% formamide, 0.1 M Tris-HCl, pH 7.5, 1mM EDTA), denatured by boiling for 2 min and incubated for 15 min at 37°C to allow self-annealing. For digestion with S1 nuclease, the cp DNA was dissolved in 0.1 × SSC, denatured, and self-annealed as above. Digestion with nuclease S1 (200U/μg DNA, 37°C, 1 h) was carried out according to Gelvin and Howell (1979).

Agarose gel electrophoresis and Southern hybridization. To detect inverted repeat sequences in cp DNA, agarose gel electrophoresis was carried out. The cp DNA digested with nuclease S1 was subjected to 0.7% agarose gel electrophoresis as described elsewhere (Yamada, 1982).

Mapping of rRNA genes on the cp DNA was performed by a double digestion of the whole cp DNA with KpnI, SstI, and XhoI. The DNA bands separated on the agarose gel were transferred onto a strip of nitrocellulose filter (Southern, 1975) and hybridized with ^{32}P-labelled pTCP 243, a plasmid that carries tobacco rRNA genes (Takaiwa and Sugiura, 1980). The location of 16S and 23S rRNA genes was determined by the hybridization with the ^{32}P-labelled BamHI 2.4 kbp fragment and SstI 3.4 kbp fragment of pTCP 243, respectively (Takaiwa and Sugiura, 1980). The DNA fragments were labelled in vitro to a specific activity of 2×10^8 cpm/μg DNA by the T4-DNA polymerase method according to O'Farrell et al. (1980). Hybridization was performed at 42°C in 50% formamide, 4 × SSC for 20 ∿ 24 h.

Electron microscopy. Spreading of the DNA, and preparing, staining, and shadowing of the grids were performed according to Davis et al. (1971). For R-loop formation, and chloroplastic rRNAs were prepared according to Galling and Jordan (1972). After electrophoresis, the SstI 10.8 kbp fragment of the cp DNA (0.2 μg) was extracted from the gel, and was mixed with rRNAs (1 μg) in a solution containing 70% formamide, 0.3 M NaCl, 0.01 M Tris (pH 8.5)

0.001 M EDTA. DNA-RNA hybridizations was based on Bedbrook et al. (1977), though the hybridization period (20 ∿ 24 h) was longer. An electron microscopic observation of the DNAs was performed by a JEM 100B (JEOL) electron microscope. Plasmid pBR 322 and ϕX 174 phage DNA were used as internal double and single strand markers, respectively.

RESULTS

Characterization of inverted repeat sequences.

To characterize the physical arrangement of *Chlorella* cp DNA, the DNA was tested for the existence of inverted repeat sequences by two methods: agarose gel electrophoresis and electron microscopy.

Figure V-1(A) shows an agarose gel electrophoresis pattern of the cp DNA that was heat-denatured, self-annealed, and then digested with nuclease S1. A single DNA band at the position of ca. 23 kbp was visualized (lane b and c), which indicates that the double strand fragment was derived from some self-annealed inverted repeat sequences in the cp DNA. This was confirmed by an electron micrograph of the self-annealed cp DNA (Fig. V-2). The characteristic "snap-back" structure consisting of two single copy-loop regions (a small loop and large loop) connected by a region of double-strands (inverted repeats) was obvious. The size of each region was: small loop, 29.5 ± 1.0 kbp (n=20); inverted repeat,

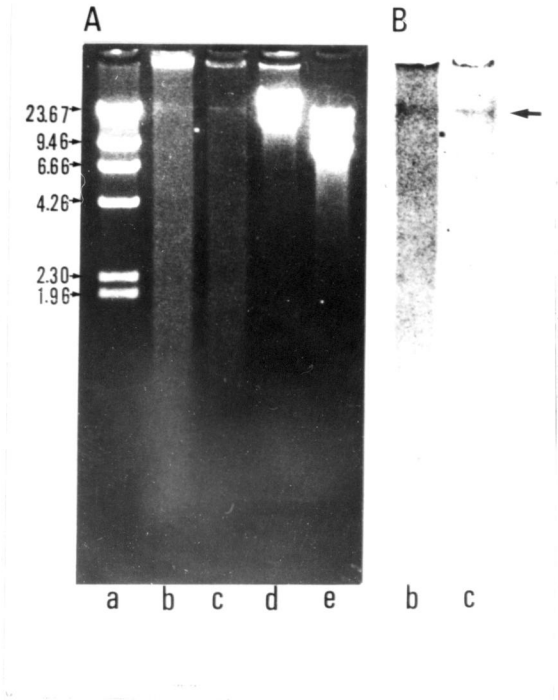


Fig. V-1. Agarose gel electrophoresis of cp DNA from C. ellipsoidea.

(A) DNA digested by S1 nuclease after self-annealing. The DNA digest, 1.0 μg (lane b) and 0.5 μg (lane c) were applied on 0.7% agarose gel. The lane a, d, and e contained λDNA digested with HindIII, XhoI, and SmaI, respectively.

(B) Hybridization of the DNA digest from lane b and c in (A) with a ^{32}P- labelled plasmid pTCP243.

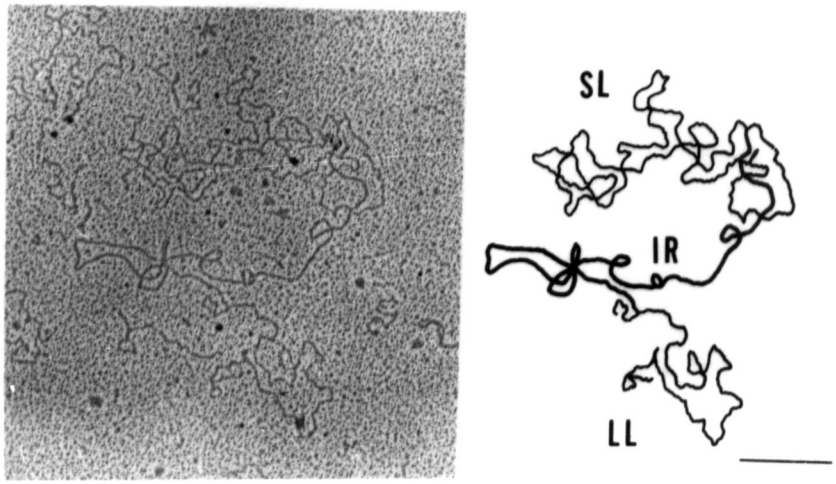


Fig. V-2. "Snap-back" structure of cp DNA from C. ellipsoidea.
A part of the large single strand loop has been lost during preparation.
SL, small loop of single strand DNA; LL, large loop of single strand DNA;
IR, inverted repeat sequences. Bar marker represents 1.0 μm.

23 ± 0.5 kbp (n=25); large loop, 98.5 ± 2.0 kbp. The size of the large loop was obtained by subtracting the sizes of small loop and of inverted repeats from the size of the whole cp DNA (175 kbp; Yamada, 1982), because its portion was usually lost during preparations.

Generally, inverted repeat sequences in plant cp DNAs are known to contain chloroplast rRNA genes (Bogorad, 1981). It was also the case for the Chlorella cp DNA; when the inverted repeats that were separated on agarose gel were transferred onto a nitrocellulose filter by the method of Southern and hybridized with plasmid pTCP 243 carrying rRNA genes from tobacco (Takaiwa and Sugiura, 1980), a specific hybridization occurred as shown in Fig. V-1(B).

Characterization of rRNA genes.

For further characterization and localization of rRNA genes, restriction fragments of the whole cp DNA produced by double digestion with KpnI, SstI, and XhoI were analysed by hybridization with the probe, pTCP 243. Figure V-3 shows the agarose gel electrophoresis pattern of the restriction fragments (A), and the hybridization patterns with the labelled tobacco rRNA genes (B, C, and D). Since the tobacco rRNA genes hybridized with only one doublet SstI fragment whose size was 10.8 kbp (B, lane c and h), this fragment must contain Chlorella rRNA genes (5S, 16S, and 23S). KpnI produced two singlet (8.49 kbp and 4.08 kbp) and two doublet hybridizing fragments (4.52 kbp and 1.08 kbp), (B, lane b and e) and XhoI

produced four singlet hybridizing fragments (21.7 kbp, 17.5 kbp, 9.76 kbp, and 4.85 kbpP, (B, lane f and i). The KpnI 4.52 kbp doublet fragments were cleaved by XhoI to 3.72 kbp and 0.8 kbp (the shorter one was lost from the gel), (B, lane g). The KpnI 4.52 kbp and 0.80 kbp fragments were inside of the SstI 10.8 kbp fragments which was revealed by double digestion with KpnI and SstI (B, lane d). The four XhoI fragments were cleaved by SstI to two doublet hybridizing fragments: 7.65 kbp and 3.26 kbp (B, lane j). From these hybridization patterns, rRNA genes were mapped as shown in Fig. V-4. The size of the inverted repeat sequences of Chlorella cp DNA was calculated to be ca. 22.5 kbp, which agrees with the value obtained from the electron microscopic study.

The orientation of 23S and 16S rRNA genes.

The orientation of 23S and 16S rRNA genes in the cp DNA was determined by the hybridization of the restriction fragments with the 3.4 kbp SstI fragment of pTCP243 that carries the tobacco 23S rRNA gene and with the BamHI 2.4 kbp fragment that carries the tobacco 16S rRNA gene (Takaiwa and Sugiura, 1980). Figure V-3C shows that the 16S rDNA hybridized with the fragments of KpnI-8.49 kbp and -4.08 kbp (lane b and e), of SstI-10.8 kbp (lane c and h), and of XhoI-9.76 kbp and -4.85 kbp (lane f and i). The double digestion with KpnI and SstI produced only one hybridizing fragment of ca. 2.45 kbp (lane d). The KpnI and XhoI double digestion (lane g) produced the same hybridizing bands as of the KpnI digestion

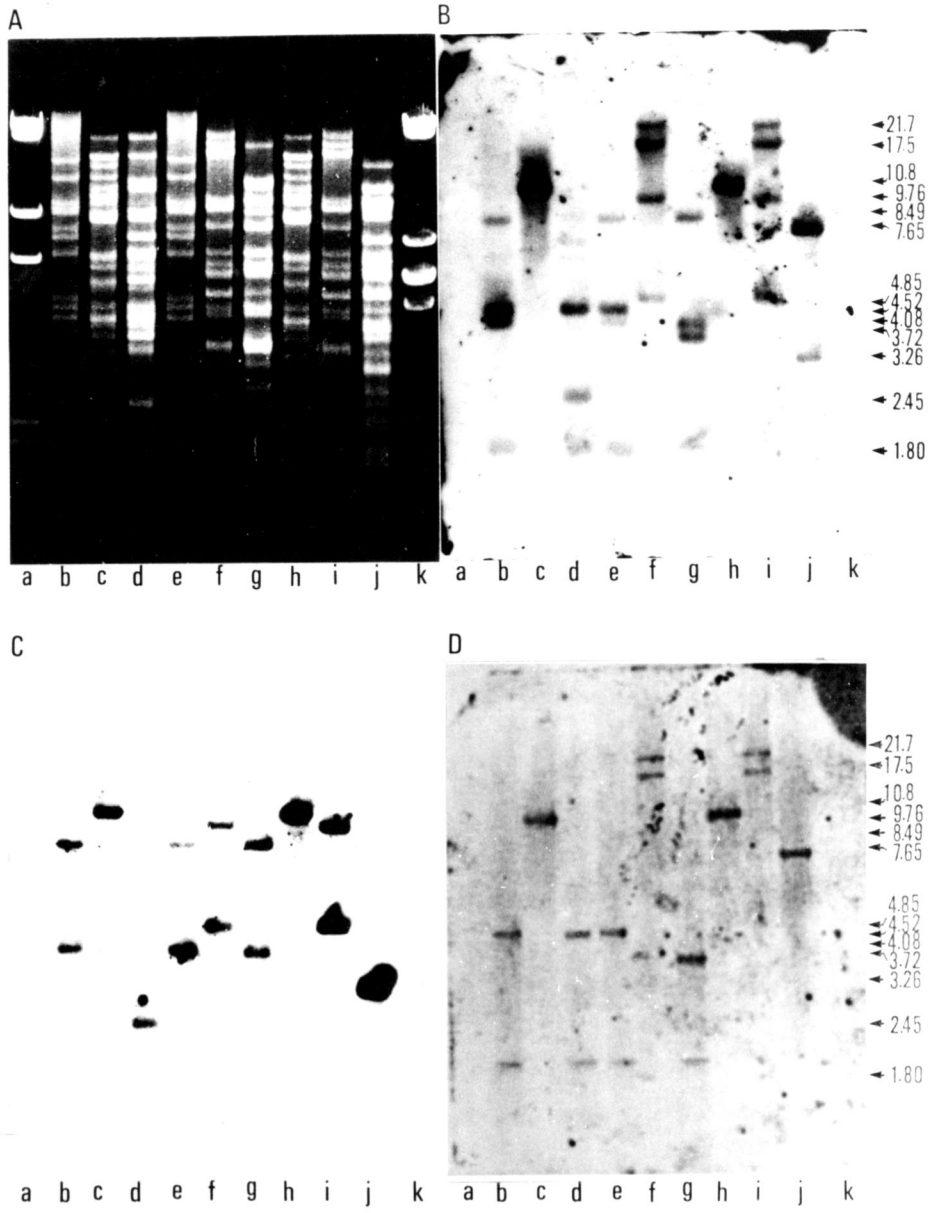


Fig. V-3. Agarose gel electrophoresis and Southern hybridization of cp DNA from C. ellipsoidea.

The cp DNA was digested with KpnI, SstI, and XhoI (A), hybridized with ^{32}P-labelled pTCP243 (B), and hybridized with ^{32}P-labelled SstI-3.4 kbp fragment of pTCP243 which carries 23S rRNA gene (C). (a), λDNA digested with HindIII; (b), KpnI; (c), SstI; (d), KpnI+SstI; (e), KpnI; (f), XhoI; (g), KpnI+XhoI; (h), SstI; (i), XhoI; (j), SstI+XhoI; (k), λDNA digested with EcoRI (all on the same o.5% agarose gel). Sizes are shown in unit of kilo base pairs.

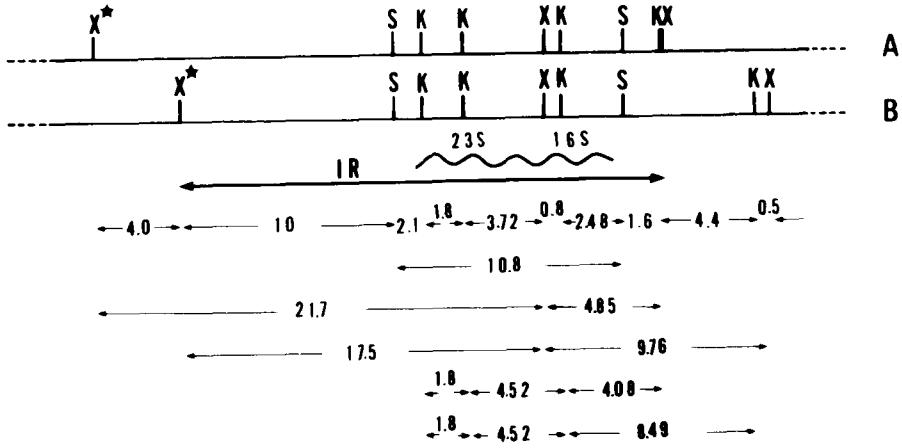


Fig. V-4. Restriction enzyme maps of rRNA genes and inverted repeat sequences of cp DNA from C. ellipsoidea.

A and B represent two counterparts which contain each repeat sequences. The wavy line and the solid line represent rRNA genes and inverted repeat sequences, respectively. Sizes are shown in unit of kilo base pairs.

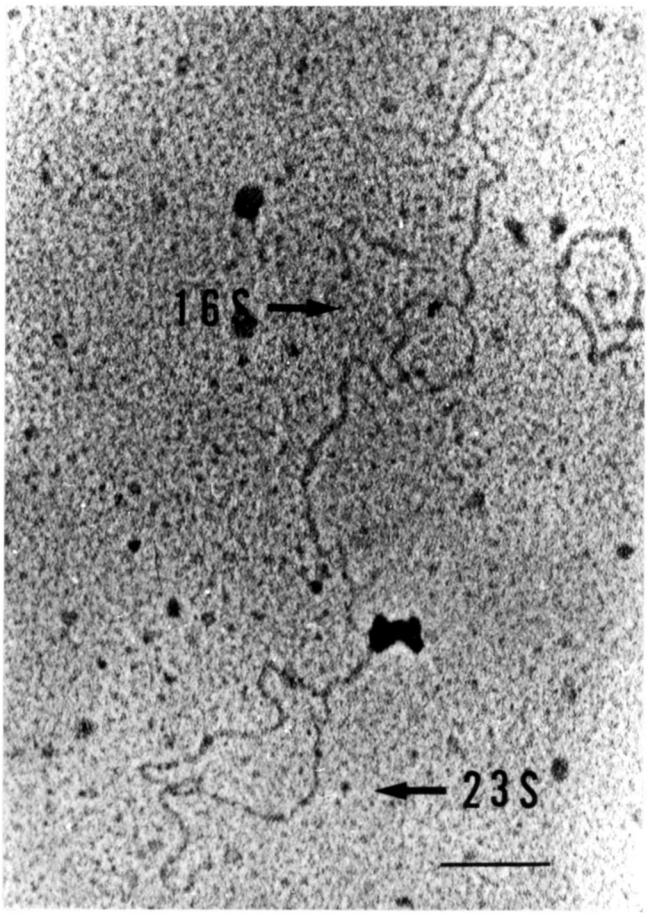


Fig. V-5. R-loop structure formed between Chlorella chloroplast rRNAs and the SstI 10.8 kbp fragment of Chlorella cp DNA. Arrows indicate 23S and 16S rRNA genes. Bar marker represents 0.5 μm.

(lane b and e) and the SstI and XhoI double digestion produced only the 3.26 kbp fragment (lane j). These patterns not only indicate the position of the 16S gene on the right hand of the rRNA gene cluster but also support the order of restriction sites mapped in Fig. V-4.

The 23S rDNA fragment of pTCP 243 hybridized with the fragment of KpnI-4.52 kbp (doublet) and -1.8 kbp (doublet) (Fig. V-3D, lane b and e), of SstI-10.8 kbp (D, lane c and h), and of XhoI-21.7 kbp and -17.5 kbp (D, lane f and i). The double digestion with KpnI and SstI (D, lane d) yielded the same hybridization pattern as with the KpnI digestion (D, lane b and e). The KpnI and XhoI double digestion produced two doublet hybridizing bands of 3.72 kbp and 1.8 kbp (D, lane g) and the SstI and XhoI double digestion produced only the 7.65 kbp fragment (D, lane j). These results suggest that the 23S gene may be at about the center of the inverted repeats (Fig. V-4).

Furthermore, the 23S and 16S rRNA genes of *Chlorella ellipsoidea* were mapped by the R-loop technique. Figure V-5 shows a typical R-loop formed between the SstI 10.8 kbp fragment and the *Chlorella* chloroplastic rRNAs; the mean sizes (n=10) of the 23S gene, the spacer, and the 16S gene were 2.46 ± 0.23 kbp, 2.93 ± 0.30 kbp, and 1.22 ± 0.18 kbp, respectively.

DISCUSSION

The large cp DNA of C. ellipsoidea also possesses a pair of inverted repeat sequences; their size (ca. 23 kbp) is comparable with those of most higher plants and is somewhat larger than that of Chlamydomonas reinhardii (19 kbp) (Rochaix, 1978). The inverted repeats of C. ellipsoidea are separated from each other by two single-copy regions (29.5 kbp and 98.5 kbp, respectively). The large single-copy region (98.5 kbp) occupies 56% of the whole cp DNA and it is a common feature among most higher plants. The small single-copy region of Chlorella (17% of total), however, considerably larger than the case for higher plants: the rations of small region to total size are 9.3% for maize (Bedbrook et al., 1977) 9.5% for wheat (Bowman et al., 1981), 8.1% for tobacco (Fluhr and Edelman, 1981), and 12% for spinach and lettuce (Kolodner and Tewari, 1979). In the cp DNA of Chlamydomonas reinhardii, the small single-copy region is almost as large as the large region (ca. 38% of total size). It is interesting that the increased size of the small loop appears to parallel the increase in genome size. Therefore the variation in the size of algal cp DNA's appears to be due to insertions and/or deletions in the small single-copy region. Both the green algal cp DNAs from Chlorella and Chlamydomonas contain large inverted repeats but the size the location of the repeats are considerably different in the two. It is interesting to reveal major rearrangements occurred through the evolution in the two

cp DNAs. For this purpose, a hybridization analysis of restriction fragments and a heteroduplex analysis between the two will be fruitful.

The chloroplast rRNA genes of C. ellipsoidea were found to be located within the inverted repeat sequences of the cp DNA (Fig. V-4). The 23S and 16S genes were finely mapped by the R-loop technique (Fig. V-5), and their sizes were found to be comparable to those of higher plants and algae so far studied (ca. 2.6 kbp for the 23S gene and ca. 1.3 kbp for the 16S gene). Since the rRNA genes in the Chlorella cp DNA hybridized efficiently with those of tobacco (pTCP 243), their nucleotide sequences must have been highly conserved.

In most higher plants, the chloroplast rRNA genes cluster in the order of 16S-23S-4.5S-5S (5'→3'); the 23S and 4.5S rRNA genes are separated by a spacer region of ca. 100 bp (Wallace, 1982). The nucleotide sequences of the 4.5S rRNA from several origins indicate their remarkable similarities to that of the 3'-terminal region of E. coli 23S rRNA (Mackay, 1981). The spacer region between 23S and 4.5S rRNA genes is suggested to be an intron (Takaiwa and Sugiura, 1982). In fact, such intervening sequences were reported in the 23S rRNA gene from Chlamydomonas reinhardii (Allet and Rochaix, 1979). It is interesting to survey the occurrence of such intron in the 23S rRNA gene of algal cp DNAs to elucidate its function and origin. R-loop structure (Fig. V-5), however, gives no indication

for the presence of such intervening sequences in the 23S gene of Chlorella. Although the 23S gene of Chlorella (ca. 2.5 kbp) is somewhat smaller than that of E. coli (ca. 2.9 kbp), there have been no evidence for the existence of 4.5S rRNA species in Chlorella chloroplasts.

It has been well documented that most cp DNAs from higher plants and algae contain tRNA genes in the rRNA operon in a similar manner to the E. coli genome (Wallace, 1982). A common feature of plant cp DNA is the presence of introns in the isoleucine and alanine tRNA genes located in the 16S-23S rRNA spacer region (Wallace, 1982). The spacer of E. coli, of Anacystis nidulans, and of Euglena gracilis chloroplast are shorter than that of most higher plants (Tomioka et al., 1981) and the tRNA genes in this region contain no introns (Tomioka, personal communication). Since the 16S-23S spacer region of Chlorella (2.9 kbp) is as large as that of higher plants, tRNA introns might also present in this alga. In Chlamydomonas, two additional rRNA genes (3S and 7S) are reported to be located in the spacer region (Rochaix and Malnoe, 1979) but there is no evidence for such genes in Chlorella.

In order to fully characterize the Chlorella cp DNA and to elucidate its evolutional relationship to those of other algae and higher plants, a physical and functional map of the Chlorella cp DNA is in the course of construction.

SUMMARY

A circular chloroplast DNA isolated from a green alga Chlorella was shown by agarose gel electrophoresis and electron microscopy to contain a pair of large inverted repeat sequences of ca. 23 kbp. The repeats were separated from each other by a small single copy region of 29.5 kbp and a large single copy region of 98.5 kbp.

Digestion with the restriction endonucleases KpnI, SstI, and XhoI, and hybridization with ^{32}P-labelled tobacco rDNAs revealed that the genes for 16S and 23S rRNAs are present in the repeated sequences. From the hybridization pattern, a restriction map around the sequences was constructed, and the rRNA genes were found to be on the 10.8 kbp SstI fragment. This location was supported by electron microscopy (R-loop formation).

The physical organization of the inverted repeat sequences and the location of the rRNA genes in the Chlorella chloroplast DNA are considerably different from those of other algae, Chlamydomonas and Euglena, and are rather similar to those of higher plants.

CHAPTER VI

CONCLUSION AND GENERAL DISCUSSION

Protoplast-Forming *Chlorella* Strains.

The existence of a rigid, indigestible cell wall in *Chlorella* cells has for a long time been a serious problem in both basic and applied microbiological researches on this alga. A method to digest the cell wall and to form biologically active protoplast was developed in this work. The method converts up to 90 % of the cells (*C. ellipsoidea* C-87) to protoplasts. This method is, however, not effective to all species of *Chlorella*, because the structure and the chemical composition of cell wall varies strain by strain. In this study, the *Chlorella* cell wall was classified into four types by using electron microscopy and the specific staining technique; (1) that with a sporopollenin layer, (2) that with an outer trilaminar layer without sporopollenin, (3) that with an outer monolayer, and (4) that without an outer layer. Protoplasts were obtained only from strains with the type(3) cell wall. All the strains which formed protoplasts in this study belong to the *Chlorella vulgaris* group (Kerfin and Kessler, 1978); those are *C. vulgaris*, *C. ellipsoidea*, and *C. saccharophila*).

Based on the data obtained in this work, a simple effective method is proposed to test *Chlorella* strains on their potentiality of protoplast formation through enzymatic digestion: cell wall of

protoplast forming strains are strongly stained with Calcofluor White and slightly with Ruthenium Red, on the other hand, strains inactive in protoplast formation are stained strongly with Ruthenium Red but slightly with Calcofluor White.

Efficiency of Protoplast Formation.

The rate-limiting factor of cell wall digestion in protoplast-forming strains was the existence in cell wall of an outermost thin layer which is probably composed of pectin. It took about eight hours to convert 80 % of *C. ellipsoidea* cells protoplasts, but the inner bulky layer of the cell wall had been almost decomposed by two hours. Some chemical or mechanical pretreatment to breach or weaken the outer layer may enhance the efficiency of the enzymatic digestion and save the time. A pretreatment of cells with several kinds of pectinase may also be effective. Since the stability of the outer region of cell wall seems to be acquired at the latest stage of cell wall formation, cells at a specific growth phase in a synchronized culture may serve as a good source of protoplasts.

The enzymes effectively used in the protoplast formation were the cellulase Onozuka, the Macerozyme, and purified pectinase; the first and the second enzymes are crude enzyme mixtures prepared from *Trichoderma viride* and from *Phizopus* sp., respectively. The fractionation, isolation, and characterization of essential agents for the cell wall digestion in these enzyme

preparations should be the subject of further study to improve the method and to prevent a deterioration of protoplasts.

Usefulness of *Chlorella* Protoplasts in Industry.

It is the rigid and indigestible cell wall that have for a long time restricted a utilization of *Chlorella* cells as a practical, industrial subject. Since a method to digest the cell wall has been found, the value of *Chlorella* in this field should increase. From the protoplasts, many useful materials such as proteins, carbohydrates, lipids, vitamins, and pigments may be isolated with less time, cost, and labor without any deterioration.

No strong evidence seems to exist that *Chlorella* in the diet of human or animal is potentially harmful, but there have been a fair weight of evidence that the fresh or dried algal cells are too resistant to allow non-ruminants to use their protein nutrients to any useful extent (Vincent, 1971). Protoplasts of this alga will be, however, used as food.

The high mass-productivity and the high protein content of *Chlorella* cells suggest utility of this organism for a biological production of specific useful proteins, using the cell as a host in the molecular cloning techniques. In addition to many indigenous advantages of *Chlorella* cells, a large body of knowledge and techniques of mass-culturing of this alga accumulated for the past 30 years will greatly advance the utility of *Chlorella* in industrial applications.

Usefulness of *Chlorella* Protoplasts in Basic Researches.

The protoplasts prepared here were shown to be biologically active by a test for the affinity to neutral red, a dye for vital staining, the ability to evolve oxygen by photosynthesis (retaining 80 % O_2 evolution of intact one, unpublished result), and the ability to regenerate cell wall and to grow. Therefore, the protoplasts can serve as a useful tool to study several aspects of biochemistry and physiology as well as genetics and molecular biology of *Chlorella*. The specific examples are as follows:

(1) Mechanism and process of cell wall regeneration and the successive cell division.

(2) Membrane transport of several substances.

(3) Action of some drugs or hormones on the membrane.

(4) Isolation of cell components.

(5) Biological activities (e.g., photosynthesis) of cellular components.

(6) Somatic hybridization for genetic analysis or breeding.

(7) Transplantation of some cellular components (chloroplast or mitochondrion).

(8) Transformation with several DNAs.

Some of these subjects were already, however in principle, realized in the present work; those are the cell wall regeneration, the protoplast fusion, and the isolation of chloroplasts and chloroplast DNA. Since no means have been so far available for the study of genetics and molecular biology of *Chlorella*, the

protoplasts must be of great use in this field.

Molecular Biology of Chloroplast of *Chlorella*.

Chloroplast-nucleus interaction.

Like higher plants, the photosynthetic apparatus of *Chlorella* consists of two photosystems I and II. Light energy is converted to chemical energy in ATP and NADPH through these photosystems. A series of biosynthetic reactions then occur via Calvin-Benson cycle. All of these fundamental reactions occur in the thylakoid membrane system and the cytosol of chloroplast under a complicated and elaborate regulation. The biosynthesis of chloroplast components depends to a large degree on the activity of the nucleo-cytoplasmic compartment, but chloroplast also possess its own protein synthesizing system which includes DNA, RNA, DNA- and RNA-polymerases, ribosomes, and several additional factors. One of the most interesting matters is the close cooperation between the organellar and the nuclear protein synthesizing systems; chloroplast enzymes consist of subunits, some of which are made within the organelle, while others are synthesized in the nucleus. The nuclear genes must be transcribed in the nucleus and the resultant mRNAs are translated in the cytoplasm on 80S ribosomes. Before the cytoplasmic subunit can associate with their chloroplast partner, these polypeptides have to traverse the chloroplast envelope. Very little is known about the mechanism of the regulation in this process. To answer this fundamental question, it is

necessary to characterize both the nuclear DNA and the chloroplast DNA in detail.

Knowledge on molecular aspects of Chlorella chloroplast DNA (cp DNA) was for the first time obtained by this work.
In addition, knowledge on nuclear genes of Chlorella will be obtained through the protoplast fusion method proposed in this work using several kinds of mutants; many kinds of mutants of Chlorella are already available (Granik and Allen, 1971).

Physical properties of Chlorella cp DNA.

Some characters of Chlorella cp DNA are compared with those of higher plants and of some algae in Table VI-1. Like higher plants and algae, cp DNA of Chlorella is a large circular molecule existing in multi copies and high in the AT content. A special feature of Chlorella cp DNA is its large size (175 ∿ 180 kbp) which is considerably larger than those of most higher plants or Euglena (ca. 150 kbp) and is comparable with that of Chlamydomonas reinhardii (190 kbp). Although the meaning of the size difference is unclear, it would imply a degree of evolutional relatedness of Chlorella to other algae or to higher plants. Since a very small cp DNA (ca. 85 kbp) is known in a green alga Codium fragile, some portion of the cp DNA of Chlorella might not always be necessary for the chloroplast function itself.

In "the endosymbiotic theory" for a chloroplast origin

Table VI-1. Sizes of circular monomers and mean G+C contents of chloroplast DNA's [a]

	Species	G+C content (%)	Mean length (μm)	Molecular weight (mega dal.)
Angiospermae				
Dicotyledons	*Antirrhinum majus*	38	46	94.5
	Beta vulgaris	38	46	94.5
	Lactuca sp.	39	41	84.2
	Oenothera hookeri	38	45	92.4
	Phaseolus sp.	39	40	82.2
	Pisum sp.	32.7-39	39	80.1
	Spinacia oleracea	37	44	90.4
	Spinacia oleracea	38	46	94.5
	Spinacia oleracea	37-39	39.5	81.1
	Tropaeolum majus	-	44	90.4
Monocotyledons	*Avena sativa*	39	37	76.0
	Narcissus pseudonarcissus	-	44	90.4
	Narcissus pseudonarcissus	37	-	-
	Tulipa gesneriana	-	43.6	89.6
	Zea mays	38	43	88.3
	Zea mays	39	38	78.1
	Spirodela oligorrluza	37	54	111.8
Archegoniatae				
Filicinae	*Asplenium nidus*	38	44	90.4
	(Pteris vittata)	38	43	88.3
Musci	*Sphaerocurpos donnellii*	31	37	76.0
Algae				
Euglenophyceae	*Euglena gracilis*	28	40/44	82.2/90.4
Chlorophyceae	*Chlamydomonas reinhardii*	36	62	126
	Acetabularia	-	-	1100-1500
	Codium fragile	37	28	56
	Chlorella ellipsoidea	36	56	115
Xanthophyceae	*Vaucheria sessilis*	38	36	73.9
	Olisthodiscus luteus	35	40-48	95
Cyanelle	*Cyanophora paradoxa* Cyanelle	52	53	110

a, According to Herrmann and Possingham (1980).

(Margulis, 1968), a free living organism similar to the present day blue-green algae invaded a primitive eukaryotic cell, and later degenerated into a chloroplast through a loss of some characters, on some genetic information. This is, in fact, observed in a well documented symbiotic association between a protozoa Cyanophora paradoxa and its cyanelles (Trench et al., 1978). The size of cyanelle DNA (ca. 174 kbp) is about a twentieth of those of most blue-green algae (Mucke et al., 1980; Klein et al., 1981) and is comparable with that of Chlorella chloroplast.

It is important and interesting to elucidate which portion of DNA is conserved and which portion is variable in cp DNAs among higher plants, algae, and cyanelles. For this purpose, cp DNA of Chlorella as well as those of higher plants and algae must be characterized in its physical and functional arrangements.

Physical arrangement of Chlorella chloroplast DNA.

The molecular arrangement of Chlorella cp DNA is compared with those of higher plants and algae in Fig.VI-1. Most cp DNAs of higher plants (monocotyledons and dicotyledons) so far studied possess a pair of long inverted repeat sequences whose size varies from 21 kbp to 28 kbp (Bedbrook and Bogorad, 1976; Kolodner and Tewari, 1979; Whitfeld et al., 1978). Chlamydomonas reinhardii contains a pair of somewhat shorter inverted repeats of 19 kbp (Rochaix, 1978). On the other hand, Euglena gracilis cp DNA contains three very short tandemly repeated sequences of 5.6 kbp each (Gray and Hallick,

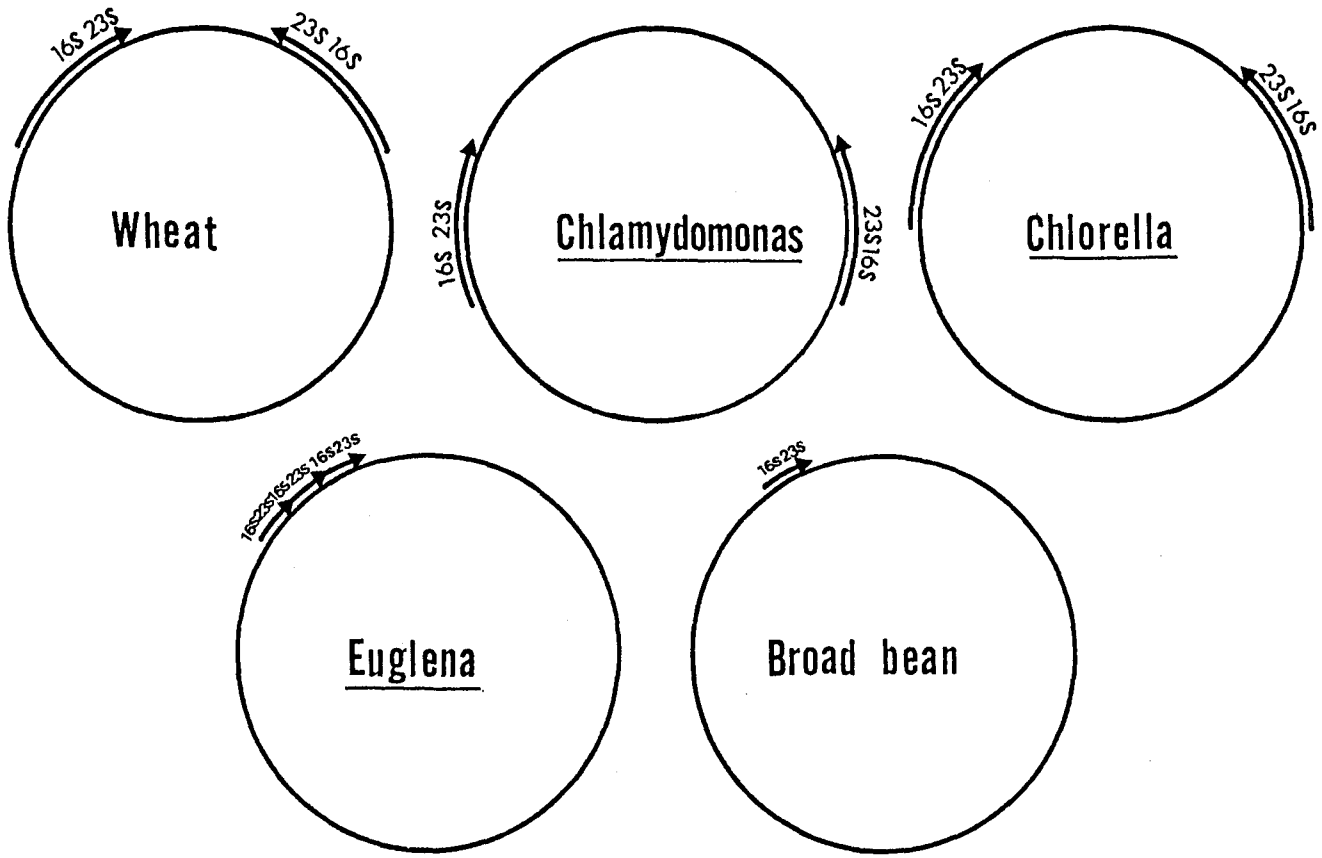


Fig.VI-1 Chloroplast DNAs from higher plants and algae

1977; Rawson et al., 1978). Some leguminous plants such as Vicia fava (Koller and Delius, 1980) and Pisum sativum (Palmer and Thompson, 1981) exceptionally lack the inverted repeat sequences in their cp DNAs.

The cp DNA of Chlorella also possess a pair of inverted repeats (ca. 23 kbp) as those of most higher plants and Chlamydomonas but its arrangement is somewhat different from those of higher plants and Chlamydomonas. On self-annealing, the cp DNA molecules of higher plants and of Chlorella form a small loop (13 ∿ 20 kbp for higher plants and ca. 30 kbp for Chlorella) and a large loop (80 ∿ 100 kbp for higher plants and Chlorella), on the other hand, cp DNA of Chlamydomonas gives two large loops (70 ∿ 80 kbp). The size of the small loop of Chlorella was between those of higher plants and that of Chlamydomonas. The DNA sequence in the small loop region seems to be highly variable among plants and algae.

The inverted repeat sequences of cp DNAs are similar in size among higher plants and algae (Table VI-2). Denaturation mapping by Kolodner and Tewari (1979) gave some homology in sequence of the inverted repeats among higher plants. Though the significance of inverted repeat sequences in cp DNA is yet unknown, Kolodner and Tewari (1979) proposed that the existence of inverted repeats might improve the stability of a repeated sequence itself as well as help maintain the similarity of the two copies of the inverted

Table VI-2. Physical Arrangement of Chloroplast DNAs

Origins	IR (kbp)	SL (kbp)	LL (kbp)
Maize	22.5	12.6	78.5
Wheat	21.0	12.8	80.2
Daffodil	28.5	16.6	87.8
Tobacco	24.0	13.0	99.0
Lettuce	24.4	19.6	87.0
Spinach	24.4	18.5	86.0
Chlamydomonas	19.7	72.4	78.5
Euglena	5.6 (tandem)		
Chlorella	23.0	29.5	98.5

IR, inverted repeat sequences; SL, small loop region; LL, large loop region.

repeat by promoting continual recombination and heteroduplex repair events between the two copies. It is not known why the inverted repeats are absent from cp DNAs of some leguminous plants (P. sativum and V. faba) or Euglena gracilis.

To elucidate which portion of cp DNA is conserved and which portion is variable among higher plants and algae, further studies such as fine physical mapping of cp DNAs or hybridization among restriction fragments of cp DNAs are necessary.

Mapping of genes for rRNAs and LS of RuBPCase.

The rRNA genes (16S and 23S) of C. ellipsoidea was mapped in the small loop end of the inverted repeats. This arrangement of rRNA genes is considerably different from that of Chlamydomonas and Euglena; in the former, rRNA genes cluster at the central part of the 19 kbp inverted repeats (Rochaix and Malnoe, 1978) and in the latter, rRNA genes are tandemly repeated three times (Rawson et al., 1978). The orientation and arrangement of rRNA genes of Chlorella is similar to that of higher plants. The rRNA genes are ordered as 16S-23S-4.5S-5S(5'→3') in higher plants (Takaiwa an Sugiura, 1980) and probably in Chlorella, on the other hand, in Chlamydomonas the order 16S-7S-3S-23S-5S(5'→3') is reported (Rochaix, 1981). Two unusual features of Chlamydomonas cp rRNA genes are noticeable; first, the gene for the 23S rRNA contains an intervening sequence of 870 bp and second, the genes coding for 3S and 7S rRNA are present (Rochaix, 1981). R-loop formation study

on Chlorella cp DNA with rRNAs of Chlorella indicated that there is no intervening sequence in either the 23S on the 16S gene (unpublished data).

Since the whole rRNA gene cluster of Chlorella is covered by the fragment SstI-10.8 kbp produced by digestion with SstI, cloning, fine mapping, and sequencing of the rRNA genes will be easily carried out.

Ribulose-1,5-bisphosphate carboxylase (RuBPCase, 4.1.1.39) which is a key enzyme for CO_2 fixation in Calvin-Benson cycle is comprised of eight identical small subunits of 12,000 ∿ 14,000 daltons together with eight identical large subunits of 50,000 ∿ 55,000 daltons (Kawashima and Wildman, 1970). The gene for the small subunit of RuBPCase was shown to be located in the nuclear genome but the large subunit (LS) of this enzyme to be located in the chloroplast genome (Bogorad, 1981). Using the labelled plasmid pSocE48 which carries the spinach LS gene (Zurawski et al., 1981), the presence of LS gene of Chlorella in the large loop region of the cp DNA was elucidated by Southern bybridization method (unpublished data). The mechanism of expression and organization of nuclear and organellar proteins will be studied with this gene.

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