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Review

The *Chlamydomonas* cell walls and their degrading enzymes

Yoshihiro MATSUDA

Department of Biology, Faculty of Science, Kobe University, Nada-ku, Kobe, 657 Japan

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The unicellular algae like *Chlamydomonas* and their related colonial members in the Volvocales are surrounded by extracellular matrices or cell walls which are composed of glycoproteins with little, if any, polyglucans characteristic of higher plant and other algal walls. Their architecture, chemistry, synthesis, secretion, *in vitro* assembly, and mutation are becoming increasingly clear particularly in the cell wall of *C. reinhardtii*, which will be briefly reviewed. Moreover, in *C. reinhardtii* a lytic enzyme (gamete wall-autolysin), which is responsible for digestion of the gamete cell wall during mating, and a hatching enzyme (sporangium wall-autolysin), which acts on the sporangium cell wall to liberate zoospores, have been purified and characterized. Recent work on the specificity, cleavage sites, intracellular storage form, location, and excretion signal of the two degrading enzymes will be discussed and their nature will be compared. Finally, discussion will be extended to some systematic approaches which employ the cell walls and their degrading enzymes as important phylogenic keys in *Chlamydomonas* and in the Volvocales.

Key Index Words: Cell wall—chemotaxonomic key—Chlamydomonas—*glycoprotein—hatching enzyme—lytic enzyme—protease—Volvocales.*

Chlamydomonas is a biflagellated, green unicell which has been used as a valuable experimental alga for almost 100 years. The genus which contains nearly 500 species is divided into 9 sub-groups (ETTL 1976, 1983), and is placed in the family Chlamydomonadaceae and the order Volvocales (BOLD and WYNNE 1985). Individual species of *Chlamydomonas* is either homothallic or heterothallic. In the homothallic strains like *C. monoica* (VANWINKLE-SWIFT and AUBERT 1983), mating can occur within clones, presumably by interconversion of the mating-type genes as observed in yeasts (HERSKOWITZ and OSHIMA 1981). Such mating-type switches, however, are absent in the heterothallic species such as *C. reinhardtii*, *C. eugametos* and *C. moewusii*, where two sexually compatible clones having either mating-type plus (mt^+) or minus (mt^-) nuclear genes exist, and no sexual reproduction occurs within clones. Copulation may occur between motile gametes which are morphologically alike (isogamy; *e.g. C. reinhardtii*, *C. eugametos*, *C. moewusii*) or morphologically different (anisogamy or heterogamy; *e.g. C. zimbabwiensis*) or between gametes differentiated as egg and sperm (oogamy; *e.g. C. suboogama*, *C. pseudogigantea*) (TSCHERMAK-WOESS 1959, 1962).

Fig. 1 shows the asexual and sexual cell cycles of an isogamous heterothallic alga, *Chlamydomonas reinhardtii*. The mt^+ and mt^- cells reproduce asexually by repeating a cycle of growth, mitosis and cytokinesis (Fig. 1A, B). When all cell divisions are complete, a new cell wall is formed around each daughter cell, and 4–16 daughter cells

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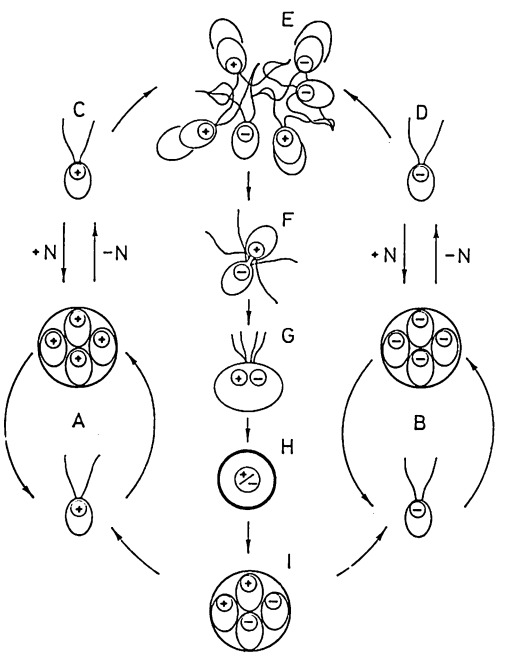

Fig. 1. The life cycle of an isogamous, heterothallic alga, *Chlamydomonas reinhardtii*. A and B, asexual reproduction of mt^+ and mt^- vegetative cells; C and D, mt^+ and mt^- gametes induced by nitrogen starvation; E, flagellar agglutination and cell wall loss after mixing the two mating-type gametes; F, pairing and protoplasmic fusion; G, quadriflagellated young zygote; H, mature zygote with thick zygotic cell wall; I, meiotic cell division.

(zoospores) are released by breaking down the mother cell wall (sporangium cell wall). The asexual cell cycle can be synchronized by the light-dark regime (Bernstein 1960; Kates and Jones 1964).

Switching to the sexual cell cycle in *C. reinhardtii* occurs when the asexually growing vegetative cells are subjected to nitrogen starvation (Sager and Granick 1954). Gametic differentiation of either mating-type can occur in the absence of cells of the opposite mating-type and without sexual cell division (Fig. 1C, D) (Schmeisser *et al.* 1973; Matsuda *et al.* 1978). At the level of the light microscope, vegetative cells and gametes are indistinguishable. At the ultrastructural and biochemical levels, however, gametogenesis in *C. reinhardtii* involves: (1) the construction of a mt^+ or mt^- mating structure for protoplasmic fusion (Friedmann *et al.* 1968; Cavalier-Smith 1975; Goodenough and Weiss 1975; Martin and Goodenough 1975; Triemer and Brown 1975b; Goodenough *et al.* 1982), (2) the synthesis of mating-type specific agglutinin molecules, their transportation to the flagellar surface for agglutination (Wiese 1965; Goodenough *et al.* 1985; Snell 1985) and their accumulation in the cell body as a reservoir of molecules (Saito *et al.* 1985; Snell 1985), and (3) the shift in the storage form of cell wall lytic enzyme for digestion of the gametic wall (Matsuda *et al.* 1987b; see later for details).

General features of the sexual cell cycle which occur between the mt^+ and mt^- gametes in heterothallic species are similar with minor exceptions and consist of the following steps (Fig. 1): the initial clumping of gametes by flagellar adhesion (Fig. 1E), pairing of gametes by firm flagellar tip-to-tip adhesion, protoplasmic fusion (Fig. 1F, G), flagellar deadhesion and retraction, zygotic wall formation (Fig. 1H), zygote maturation, meiosis, cytokinesis (Fig. 1I), and ultimately the liberation of zygospores (4 or more) by breaking down the thick zygotic cell wall (Snell 1985; van den Ende 1985). In *C. reinhardtii*, flagellar agglutination triggers the excretion of a cell wall lytic enzyme so that paired gametes are naked before fusion (Cavalier-Smith 1975; Goodenough and Weiss 1975; Triemer and Brown 1975b; Matsuda *et al.* 1978; Goodenough *et al.* 1982; Weiss 1983). In *C. eugametos* and *C. moewusii*, the gamete wall is partially broken down at the apical end, and through the resulting hole, a plasma tube elongates towards that of its partner with which it eventually fuses. The pair joined by a narrow protoplasmic bridge, which is referred to as a "vis-a-vis" pair, swims about for several hours, and then the rest of the gamete wall is released to allow complete cell fusion (Brown *et al.* 1968; Triemer and Brown 1975a; Homan *et al.* 1980; Musgrave *et al.* 1983).

Several surveys on asexual and sexual reproduction in *Chlamydomonas* have recently been made by many authors (Wiese 1984;

ADAIR 1985; SNELL 1985; VAN DEN ENDE 1985; MATSUDA and SAITO 1986; TRAINOR and CAIN 1986; BLOODGOOD 1987). In this article, I will therefore concentrate on information on the *Chlamydomonas* cell walls and their degrading enzymes and discuss how the synthesis and degradation of cell walls are regulated temporally and spatially in the life cycle. The rest of this review will discuss some new approaches where the cell walls and degrading enzymes are used to systematize a variety of organisms in *Chlamydomonas* and in the Volvocales.

I. Cell Wall

The following information on the *Chlamydomonas* cell wall has been obtained mainly by using the isogamous, heterothallic species, *C. reinhardtii*.

1. *Vegetative cell wall*

The vegetative cell wall of *Chlamydomonas* covers the cell surface except for that of the flagella. Therefore, the wall is not continuous at the opening through which the flagella protrude: the two tunnels in the cell wall are lined by flagellar collars, cylinders of wall material (ROBERTS *et al.* 1975; SNELL 1983). The vegetative cell wall is composed of glycoproteins with none of the polysaccharide polymers characteristic of higher plant walls (ROBERTS *et al.* 1985b). The protein portion accounts for about 30% of the wall by mass and is rich in hydroxyproline to which oligosaccharides are attached (MILLER *et al.* 1972; ROBERTS *et al.* 1972). The carbohydrate portion which accounts for about 47%, contains galactose, arabinose and mannose as the most abundant sugars (MILLER *et al.* 1972; ROBERTS 1974; CATT *et al.* 1976), and is modified by sulphation (ROBERTS *et al.* 1980). Flagellar collars are different from the rest of the wall in both structure and chemical composition (ROBERTS *et al.* 1975).

Ultrastructurally, the intact cell wall was originally described as having seven layers, numbered W1–W7 (Fig. 2A) (ROBERTS *et al.* 1972). However, recent images (Fig. 2B) obtained by the quick-freeze deep-etch technique (GOODENOUGH and HEUSER 1985) indicate that W3 and W5 are actually spaces. The innermost (W1) and outermost (W7) layers constitute a "warp", while the central triplet layers, which are composed of an inner amorphous layer (W2), a medial granular layer (W4) and an outer crystalline layer (W6), form a "weft". The outer crystalline layer is further divided into inner (W6A) and outer (W6B) sublayers (Fig. 2B). The W4 and W6 layers can be solubilized by chaotropic salts (*e.g.* sodium perchlorate, lithium chloride), whereas the inner layers, W1 and W2 cannot (DAVIES 1972; HILLS 1973; ROBERTS 1974; HILLS *et al.* 1975; GOODENOUGH and HEUSER 1985). The salt-solubilized fraction is

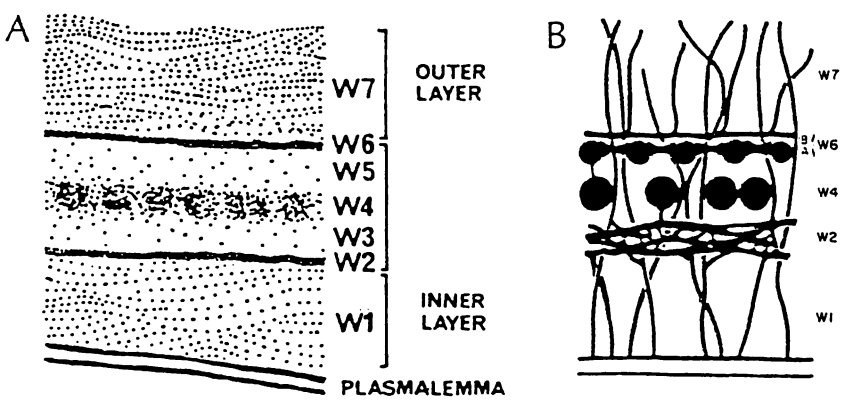

Fig. 2. Multilayered structure of the *C. reinhardtii* cell wall. A, diagram from ROBERTS *et al.* (1972); B, diagram from GOODENOUGH and HEUSER (1985).

further separated by gel filtration on Sepharose 2B into two fractions, 2BI which derives from W4, and 2BII which derives from W6 (CATT *et al.* 1976, 1978; ROBERTS 1981; GOODENOUGH *et al.* 1986). The salt-insoluble fraction can be seen under phase contrast microscopy as an extremely thin wall-shaped structure (HILLS *et al.* 1975). The salt soluble and insoluble fractions have very similar amino acid and sugar compositions (HILLS *et al.* 1975), but are composed of distinctly different species of polypeptides and glycopolypeptides in SDS-PAGE (IMAM *et al.* 1985; MATSUDA *et al.* 1985). The salt-soluble fraction contains four major fibrous glycopolypeptides (>350, 270, 150, 130 kDa) that make up 2BII subfraction (CATT *et al.* 1976; ROBERTS *et al.* 1985a; GOODENOUGH *et al.* 1986) and several minor polypeptides, whereas the insoluble fraction is composed of several polypeptides among which a 100 kDa polypeptide is prominent (GOODENOUGH and HEUSER 1985; IMAM *et al.* 1985; MATSUDA *et al.* 1985). In *C. eugametos* the cell wall contains two major glycoproteins and several minor glycoproteins (MUSGRAVE *et al.* 1983; ADAIR *et al.* 1987).

When the salt-soluble and insoluble fractions are mixed and dialyzed against water, a complete cell wall is reconstituted, as judged by light and electron microscopy (HILLS 1973; HILLS *et al.* 1975). The 2BII subfraction itself, when dialysed against water, can self-assemble into a crystalline lattice structure while 2BI cannot (HILLS 1973; HILLS *et al.* 1975; CATT *et al.* 1978; GOODENOUGH *et al.* 1986). Oligosaccharide residues, especially the terminal mannose residues of 2BII glycoproteins, are important for the self-assembly process (CATT *et al.* 1978; O'NEILL and ROBERTS 1981). If 2BI is added to 2BII and dialysed, the former is incorporated into the lattice structure, resulting in the formation of sandwiches of W6–W4–W6 layers, but they are still fragments (GOODENOUGH *et al.* 1986). Thus, the presence of the salt-insoluble fraction (W1 and W2) is essential for the reconstruction of a complete cell wall having W1–W2–W4–W6 layers (HILLS 1973; HILLS *et al.* 1975; ADAIR *et al.* 1987). The inner wall layer (W2) is considered to serve as a template onto which glycoproteins of the outer crystalline layers specifically bind (HILLS *et al.* 1975; ADAIR *et al.* 1987). The cell wall-less mutant strains, *cw*-2 and *cw*-18, are deficient in some aspects of the inner wall layer, and continue to shed the crystalline wall components into the medium (HILLS *et al.* 1975; LANG and CHRISPEELS 1976; MONK *et al.* 1983; ADAIR *et al.* 1987).

The inner wall layer is the first component to be laid down around the plasma membranes of naked daughter cells after cell divisions in the asexual cell cycle (VOIGT 1986; GRIEF and SHAW 1987). Glycoproteins of the outer layers are synthesized in the endoplasmic reticulum, glycosylated in the Golgi stack, transported to the cell surface directly or *via* the contractile vacuole, and then self-assembled onto the inner wall layer (GRIEF and SHAW 1987). When vegetative cells are cultured synchronously, turnover of the inner wall layer occurs during the cell growth period (VOIGT 1985a). It has been assumed that extension of the cell wall occurs by cleaving some cross links of the inner wall layer and incorporating additional components (VOIGT 1985a).

2. *Gametic cell wall and mother cell wall*

Differentiation of non-synchronized vegetative cells into gametes occurs under nitrogen starvation with no apparent cell division (SCHMEISSER *et al.* 1973; MATSUDA *et al.* 1978, 1987b). Moreover, vegetative cells separate their cell walls (which then become the sporangium cell walls) from the protoplasts during the initial stage of cell divisions (HARPER and JOHN 1986). Therefore, both gamete cell wall and sporangium wall are actually the vegetative cell wall itself. In fact, the cell walls and their salt-soluble extracts from vegetative cells, gametes and sporangia have very similar glycoprotein compositions on SDS-PAGE

(DAVIES 1972; MUSGRAVE *et al.* 1983; VOIGT 1985b). However, a conformational change in the vegetative cell wall seems to occur during the mitotic cell division period. MUSGRAVE *et al.* (1983) have reported that an antiserum raised against one of the major cell wall glycopolypeptides of *C. eugametos* does not react with the intact wall of vegetative cells but bind to the sporangium cell walls, suggesting that the antigenic sites become exposed in the mother cell wall due to the conformational change. Moreover, SCHLÖSSER (1976) found that a hatching enzyme, which is responsible for digestion of the mother cell wall to release daughter cells (see below), does not act on the vegetative or gametic cell walls.

3. *Zygote wall*

The zygote wall differs from the vegetative wall in both chemical composition and architecture. It consists of 60–70% sugar and 5–10% protein rich in hydroxyproline (CATT 1979; GRIEF *et al.* 1987). Glucose, present only in trace amounts in vegetative cell walls, is the principal sugar in zygote walls and accounts for about half of the sugar residues (CATT 1979). The presence of a homopolymer, β-1,3 glucan is indicated by GRIEF *et al.* (1987), and this polyglucan appears to surround the fibrous hydroxyproline-rich layer. Chaotropic agents which can solubilize the outer layers of the vegetative cell wall do not solubilize the zygote wall (CATT 1979). Structurally, zygospores are surrounded by primary and secondary walls. The primary wall is formed soon after gametic fusion. Zygote specific glycoproteins are synthesized, and become associated with fibers of the early cell walls in young zygotes, but are also secreted into the culture medium (MINAMI and GOODENOUGH 1978). The secondary wall develops inside the primary wall during the maturation period, which requires several days. It is thick and composed of two layers, the outer of which is highly convoluted and appears orange under light microscope (BROWN *et al.* 1968; CAVALIER-SMITH 1976).

II. Cell Wall Degrading Enzymes

1. *Lytic enzyme (gamete wall-autolysin)*

Chemical nature

In *C. reinhardtii* and many other species of *Chlamydomonas* (*C. iyengarii*, *C. indica*, *C. chlamydogama*, *C. gymnogama*, *C. media*, *C. zimbabwiensis*), the protoplast escapes from its enclosing gametic cell wall (Fig. 1E) during mating as a necessary prelude to cell fusion (MITRA 1949; BOLD 1949; DEASON 1967; CLAES 1971; MILLER *et al.* 1974; CAVALIER-SMITH 1975; HEIMKE and STARR 1979). Gametes of *C. reinhardtii* slip out of their cell walls by breaking down the apical region and concurrently excrete into the culture medium a cell wall lytic factor (CLAES 1971), referred to as "gamete wall-autolysin" (SCHLÖSSER 1976) or "cell wall lytic enzyme" (TAMAKI *et al.* 1981). The lytic enzyme in the medium continues to disintegrate the stripped walls and also attacks other walled gametes from the outside.

Lytic enzyme can dissolve the cell walls at all stages of the life cycle (Fig. 1; vegetative cell, gamete and sporangium) with the exception of the zygote wall (CLAES 1971; SCHLÖSSER 1976). Therefore, bioassays for lytic activity measure either the formation of protoplasts from walled cells (CLAES 1971; KASKA and GIBOR 1982; SNELL 1982) or the liberation of daughter cells from sporangia (SCHLÖSSER 1976; TAMAKI *et al.* 1981), the latter assay being 20–40 times as sensitive as the former assay (MATSUDA *et al.* 1984). The enzymatic nature of gamete wall-autolysin was first indicated by SCHLÖSSER (1976) who showed that it is heat labile, non-dialysable, precipitated from the mating medium with ammonium sulfate and inactivated with $HgCl_2$, EDTA and papain. TAMAKI *et al.* (1981) developed procedures for a sensitive and quantitative assay of lytic activity using glutaraldehyde-fixed sporangia as substrate.

They then developed a method for the purification of lytic enzyme from the mating medium under stabilized conditions. Subsequently, MATSUDA *et al.* (1984, 1985, 1987b) succeeded in purifying and characterizing the enzyme (Table 1). The purified enzyme is a single glycoprotein with a molecular mass of 62–65 kDa. Activity is inhibited by metal ion chelators (EDTA, CDTA, EGTA, α,α'-dipyridyl and 1,10-phenanthroline), SH-blocking agents (*p*-chloromercuribenzoic acid, $HgCl_2$, iodoacetate, diethyl pyrocarbonate and copper acetate), certain amino acids, α_2-macroglobulin (a protease inhibitor) and phosphoramidon (a metalloprotease inhibitor). However, lytic enzyme is insensitive to the inhibitors of serine, thiol, and carboxyl proteases (phenylmethylsulfonyl fluoride (PMSF), ε-aminocaproic acid, pepstatin A, antipain, chymostatin, leupeptin and E-64). These inhibitor specificities, together with the finding that the purified enzyme contains zinc, led MATSUDA *et al.* (1985) to conclude that cell wall lytic enzyme (gamete wall-autolysin) is a metalloprotease.

Lytic enzyme digests casein as analyzed by SDS-PAGE (MATSUDA *et al.* 1985) and reversed phase chromatography (MATSUDA *et al.* unpublished data). In order to determine the polypeptide linkages cleaved by lytic enzyme, we are currently analysing points of cleavage in enzyme-treated peptides with known amino acid sequence. Our results show that lytic enzyme does not cleave the oxidized B chain of insulin, β-endorphin or α-mating factor, but does split the peptide bonds of Phe^4-Leu^5 in dynorphin and Pro^{11}-Tyr^{12} in neurotensin (MATSUDA *et al.* unpublished data).

Wall digestion

Recent studies from three laboratories (GOODENOUGH and HEUSER 1985; IMAM *et al.* 1985; MATSUDA *et al.* 1985) have independently concluded that lytic enzyme acts on only the salt-insoluble fraction, that is, the inner wall layer (W2) of the *C. reinhardtii* cell wall. IMAM *et al.* (1985) and MATSUDA *et al.* (1985) observed by phase contrast microscopy that the "ghost" like structure of the salt-insoluble wall becomes completely invisible after lytic enzyme treatment. MATSUDA *et al.* (1985) showed by SDS-PAGE analysis that lytic enzyme does not act on any of the major or minor polypeptides of the salt-soluble fraction, but does degrade some polypeptides of the salt-insoluble fraction. GOODENOUGH and HEUSER (1985) observed by electron micro-

Table 1. Properties of cell wall degrading enzymes in *Chlamydomonas reinhardtii*.

Property	Lytic enzyme	Hatching enzyme
Molecule	glycoprotein	glycoprotein
Molecular mass		
gel filtration	65 kDa	114 kDa
SDS-PAGE	62 kDa	125 kDa
pH optimum	7.5	9.0
Temperature optimum	35°C	35°C
Isoelectric point	6.5	>10.0
Wall digestion	mother cell wall vegetative cell wall gametic cell wall	mother cell wall
Inhibitors of		
serine protease	insensitive	sensitive
metalloprotease	sensitive	sensitive
thiol protease	sensitive	sensitive
acid protease	insensitive	insensitive

scopy that as the W2 layer is degraded by lytic enzyme, fibrous units, shaped like "fishbones", are observed in the medium. Since the prominent 100 kDa polypeptide in the salt-insoluble fraction appears not to be degraded by the enzyme (IMAM *et al.* 1985; MATSUDA *et al.* 1985), it may constitute the "fishbones", while lytic enzyme may attack some core polypeptide(s) which interconnects the "fishbones". If the core or framework is degraded by lytic enzyme, the whole assemblage of the cell wall may break down.

Enzyme topography

Is lytic enzyme synthesized *de novo* during gametic induction or is it already stored in the vegetative cell? The pioneering work on the lytic factor by CLAES (1971) has shown that lytic activity found in the mating medium can also be detected in vegetative cells and gametes which have been broken by sonication. The same author also reported (CLAES 1977) that cell homogenates obtained using a French press yielded no activity unless they were subjected to sonication. Recently, we (MATSUDA *et al.* 1987b) confirmed CLAES's preliminary but suggestive findings through biochemical studies and presented some important topographic aspects of lytic enzyme in cells. Both vegetative cells and gametes contain lytic enzymes, but their storage forms are quite different (Fig. 3). In vegetative cells, lytic enzyme is stored in an inactive and insoluble form (V-form). The activation and solubilization of V-form enzyme occur either when vegetative cell homogenates from the French press are subjected to sonication or when the cells are freeze-thawed prior to homogenization. In contrast, the lytic enzyme is always found in gametic cell homogenates in an active and soluble form (G-form). When the V-form enzyme is activated and then purified, it is a glycoprotein with an apparent molecular mass of 67 kDa by gel filtration and 62 kDa by SDS-PAGE, and is sensitive to metal ion chelators and SH-blocking agents. These

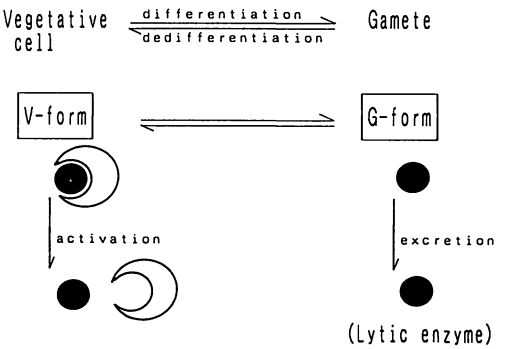

Fig. 3. Schematic diagram of the storage form of lytic enzyme in vegetative cell and gamete, and its processing during gametic differentiation and dedifferentiation.

properties are very similar to those of the G-form enzyme purified from gametic cell homogenates and lytic enzyme isolated from the mating medium. The inactive V-form enzyme was also purified by sucrose gradient centrifugation and gel filtration, and characterized as a homologous particle with a sedimentation coefficient of about 20 S (ONO *et al.* 1987). After sonication, the 20-S particle releases an active, 4-S enzyme, which coincides with the S-value of the G-form enzyme and lytic enzyme excreted into the medium (ONO *et al.* 1987). It is possible that the activity of the V-form enzyme is concealed by forming an enzyme-masking protein complex (Fig. 3) or enzyme aggregates.

It is noteworthy that the storage form of lytic enzyme shifts back and forth between the two, dependent upon gametic differentiation and dedifferentiation of the cell (MATSUDA *et al.* 1987b) (Fig. 3). When vegetative cells are starved of nitrogen, the storage form shifts from the V-form to the G-form in correlation with the ability to mate. Adding nitrogen to the gametic culture converts the G-form to the V-form, concurrently with the loss of mating ability. Therefore, we can clearly distinguish between vegetative cells and gametes, which are morphologically very similar in *C. reinhardtii*, by a simple determination of lytic activity in the cell homogenates. Without this assay, it is especially difficult to dis-

tinguish between the two cell types in agglutinin-deficient cells or flagella-less cells (SAITO *et al.* 1988; MATSUDA *et al.* 1988b).

Several pieces of indirect evidence suggest that lytic enzyme is stored in the periplasmic space (MILLIKIN and WEISS 1984; MATSUDA *et al.* 1987b). MILLIKIN and WEISS (1984) have analyzed the binding of FITC or ferritin labeled concanavalin A (Con A) to gametes by epifluorescent and electron microscopy, and proposed that Con A binding globules in the anterior periplasm of gametes represent lytic enzyme or a precursor of the enzyme. MATSUDA *et al.* (1987b) have shown that when vegetative cells and gametes are treated with exogenously added lytic enzyme, the protoplasts obtained contain little enzyme activity, and when the protoplasts are incubated further after washing out the added enzyme, lytic enzyme is again accumulated in these cells when most of the wall has been regenerated. Gametes of many wall-less mutant strains have little, if any, lytic enzyme activity in the cell homogenates and release practically no lytic enzyme into the medium during mating (MATSUDA *et al.* 1987b).

Excretion signal

A study of the release of lytic enzyme during mating is complicated by the fact that unmated gametes of some strains often release the enzyme activity, stimulated by an as yet unknown signal, and lose their walls before mixing (FRIEDMAN *et al.* 1968; GOODENOUGH and WEISS 1975; MATSUDA *et al.* 1978; MATSUDA 1980; KASKA and GIBOR 1982). Since gametes of both mating-types possess equal amounts of active (G-form) enzyme, it is possible that there is an inhibitor(s) in walled gametes (Fig. 1C, D), which acts on the cellular enzyme to keep the activity below the critical level until its release is stimulated by mixing gametes of the opposite mating-type (MATSUDA *et al.* 1987b). Some gametes might lose their walls before mixing by raising the lytic activity beyond the critical level; we have the experience that wall loss often occurs when the gametogenesis is accompanied with cell division (MATSUDA 1980) or when gametes are cultured for a long period under nitrogen-starvation.

When walled gametes of both mating-types are mixed together, lytic enzyme is released into the medium as a pulse, within 1–3 min of mixing (SNELL 1982; MATSUDA *et al.* 1987b). Both mating-types release lytic enzyme when agglutinated with either live gametes, fixed gametes, or isolated flagella of the opposite mating-type (CLAES 1971; GOODENOUGH and WEISS 1975; KASKA and GIBOR 1982). However, several reports have indicated that the mt^+ gametes release much more lytic enzyme than the mt^- gametes during flagellar agglutination (MATSUDA *et al.* 1978; SNELL 1982; MILLIKIN and WEISS 1984).

Lytic enzyme is secreted only after flagellar agglutination: Deflagellated gametes neither agglutinate nor excrete enzyme. However, agglutinability recovers when about 10% of the complete flagella is regenerated, while the ability to release the enzyme is regenerated only when 50% of the flagellar length is restored (RAY *et al.* 1978). It is thought that flagellar agglutination sends a signal to the cell body that triggers the release of the lytic enzyme (GOODENOUGH 1977). Several reports have indicated that cyclic AMP (cAMP) and Ca^{2+} ions may play a role as second messengers in signalling secretion of lytic enzyme and other events required for cell fusion. A transient 10 to 30-fold elevation of intracellular cAMP levels is observed during initial agglutination in *C. eugametos* (PIJST *et al.* 1984) and *C. reinhardtii* (PASQUALE and GOODENOUGH 1987). BLOODGOOD and LEVIN (1983) have observed that the rate of efflux of Ca^{2+} from gametes also increases up to 20 times during the initial mating, but the efflux rate returns to the control level within a few minutes. The transient increase in Ca^{2+} efflux may reflect a transient increase in the cytoplasmic free-Ca^{2+} concentration released from intracellular storage sites (BLOODGOOD and LEVIN 1983).

KASKA *et al.* (1985) used X-ray microanalysis to analyze the intracellular distribution of Ca^{2+} and revealed that Ca^{2+} is sequestered in descrete granules within the gametic cell body prior to mating only to become diffuse throughout the cell during mating. The presence of calmodulin has been reported in the cell bodies and flagella (GITELMAN and WITMAN 1980), and inhibitors of calmodulin, trifluoperazine and W-7, prevent the transduction of signals for cell wall loss (DETMERS and CONDEELIS 1986). Lidocaine, an inhibitor of the movement of Ca^{2+} ion across cell membranes, also prevents cell wall loss (SNELL *et al.* 1982). More recently, PASQUALE and GOODENOUGH (1987) reported that unmated gametes, but not vegetative cells, can be induced to undergo wall loss by addition of dibutyryl-cAMP and/or cyclic nucleotide phosphodiesterase inhibitors (*e.g.* isobutylmethylxanthine).

2. *Hatching enzyme* (*sporangium wall-autolysin*)

Chemical nature

The release of daughter cells by breaking down the surrounding mother cell wall is mediated by a second type of enzyme, named "sporangium wall-autolysin" or "hatching enzyme" (SCHLÖSSER 1966, 1976; MIHARA and HASE 1975). This enzyme is excreted into the medium as the zoospores hatch, and therefore can be concentrated from the medium of synchronized cultures (SCHLÖSSER 1976). Hatching enzyme is specific for mother cell wall and will not affect vegetative or gamete cell walls (SCHLÖSSER 1976). JAENICKE's group (JAENICKE and WAFFENSCHMIDT 1981; JAENICKE *et al.* 1987) purified the hatching enzyme of *C. reinhardtii*, and characterized it as a serine protease with a molecular mass of 37 kDa (gel filtration) or 40 kDa (SDS-PAGE) and a pH-optimum at 8.2. To compare the properties of hatching enzyme with those of lytic enzyme, we recently purified and characterized the hatching enzyme (KOSEKI *et al.* 1987; manuscript in preparation) (Table 1). Our purified enzyme specifically acts on sporangium cell walls, and is an extremely basic glycoprotein which binds to Con A-Sepharose. It has a molecular mass of 114 kDa in gel filtration and 125 kDa in SDS-PAGE, a pH-optimum at around 9.0, and is inactivated by PMSF, TLCK, $HgCl_2$, iodoacetate, 1,10-phenanthroline and EDTA. Therefore, the molecular size of our hatching enzyme is much greater than that of JAENICKE and WAFFENSCHMIDT (1981) on both gel filtration and SDS-PAGE. We noted that a protein component of about 40 kDa is one of the prominent contaminants in crude and partially purified enzyme preparations; this component might be related to the hydroxyproline-rich glycoproteins which are liberated during hatching of the zoospores from the mother cell wall (VOIGT 1985a, b). To confirm that the hatching enzyme is actually a larger molecule than the lytic enzyme, the two purified enzymes were mixed and applied to a gel filtration column packed with Sephacryl-S200. The activity, as determined by use of glutaraldehyde-fixed sporangia as substrates, gave two peaks: the heavier one (PMSF sensitive, 115 kDa peak) is hatching enzyme, and the lighter one (PMSF insensitive, 65 kDa peak) lytic enzyme (Fig. 4).

Hatching enzyme digests α-casein. The chromatographic patterns of enzyme digests

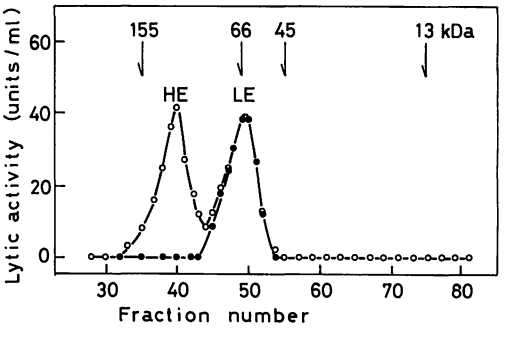

Fig. 4. Gel filtration of the mixture of the purified lytic enzyme (LE) and hatching enzyme (HE). ○, cell wall degrading activity measured by glutaraldehyde-fixed sporangia as substrates; ●, activity measured in the presence of 0.2 mM PMSF.

of α-casein, as analyzed by reversed phase chromatography, are distinctly different for hatching enzyme and lytic enzyme. Hatching enzyme hydrolyzes α-mating factor, α-neo-endorphin, dynorphin, neurotensin, mastoparan and β-endorphin, but does not act on oxidized insulin B. In general, the imino side of a Lys or Arg residue in the P_1' position is selectively cleaved, provided that the P_1 position is occupied by a basic or hydrophobic amino acid (MATSUDA *et al.* manuscript in preparation).

Intracellular enzyme

Hatching enzyme of *C. reinhardtii* seems to be formed in the young zoospores during ripening of sporangia (MIHARA and HASE 1975). Cells at later stages of the cell cycle contain the hatching enzyme, but its action is suppressed until the regular time of zoospore liberation in the cell cycle. However, if the cells are subjected to vibration, they liberate zoospores and concurrently excrete the enzyme into the medium (MIHARA and HASE 1975). In order to see the production or activation of hatching enzyme in the cells during ripening of the sporangia, we prepared cell homogenates using the French press and analyzed the activity (MATSUDA *et al.* 1988a). Unexpectedly, no activity was detected in the homogenates of sporangia even just before hatching. The homogenates were found to contain inhibitor(s) which inactivated specifically and irreversibly the activity of the isolated hatching enzyme. Therefore, it seems to be difficult to analyze the intracellular hatching enzyme through cell breakdown (MATSUDA *et al.* 1988a).

III. Systematic Studies

Many species of the genus *Chlamydomonas* have been divided into subgenera or subgroups generally based on the light-microscopically visible features of vegetative cells (PASCHER 1927; GERLOFF 1940; ETTL 1976). However, the problems of the taxonomy of *Chlamydomonas* at the species level have been presented by LEWIN (1975) who stated: 'Specific distinctions in this genus are generally based on relatively few microscopically visible features of the biflagellate (presumably haploid) cells, as found in nature or when grown in media often insufficiently characterized.··· Therefore, the taxonomy of the genus is in a mess, and little confidence can be placed on specific identifications based on published descriptions' (*cf.* TRAINOR and CAIN 1986). To overcome the confusion and artificiality of the taxonomy of this primitive green alga, several authors have considered that works on the specificity of the cell walls and their degrading enzymes might contribute a truer and more reliable assessment of natural relationships (ROBERTS 1974; SCHLÖSSER 1984; MATSUDA *et al.* 1987a).

1. *Cell wall as a taxonomic marker*

All members of the Volvocales except the family Polyblepharidaceae have cell walls or extracellular matrices which are constructed in a similar manner (BOLD and WYNNE 1985; ROBERTS *et al.* 1985a): they consist of a stable amorphous inner wall layer and a crystalline outer wall layer, and are built up of unique glycoprotein molecules with little, if any cellulose or chitin, which is abundant in most other algal cell walls. The detailed cell-wall structures and chemical compositions, however, are suggested to be different between individual algae, and could be used as an important phylogenic marker (ROBERTS 1974). ROBERTS (1974) and ROBERTS *et al.* (1982) have analyzed the glycoprotein arrays on the outer surface of cell walls in a large number of organisms of the Volvocales, and concluded that their crystal structures fall into 4 general classes (Tables 2 and 3). Eleven species of *Chlamydomonas* (*e.g. C. eugametos*, *C. moewusii*, *C. chlamydogama*) and many other members in the family Chlamydomonadaceae (*e.g. Carteria*, *Chlorogonium*, *Haematococcus*) possess the simplest cell wall crystal structure (class II). In contrast, *C. reinhardtii*, *C. cribrum*, *C. angulosa* and *C. inepta* have more complicated

Table 2. Grouping of algae in the genus *Chlamydomonas* on the basis of cell walls and their degrading enzyme specificities.

Species	SCHLÖSSER (1976, 1984)	ROBERTS *et al.* (1982)	MATSUDA *et al.* (1987a)
Chlamydomonas reinhardtii	1	I	A
C. globosa	1		A
C. incerta	1		A
C. smithii	1		A
C. cribrum		I	A
C. komma	2		A
C. angulosa	2	I	B
C. debaryana	2		C
C. inepta	2	I	
C. asymmetrica	3	III	C
C. gloeopara	3		
C. peterfii	3		
C. oblonga	4		
C. mexicana	4		C
C. iyengarii	5		
C. sphaeroides	5		
C. callosa	6		
C. aggregata	7		C
C. humicola	7		
C. applanata	7	II	
C. dysosmos	7	II	
C. culleus	9		
C. elliptica	9		
C. frankii	9		C
C. gymnogama	10		
C. segnis	10		
C. pallidostigmatica	10		
C. gelatinosa	11		
C. eugametos	12	II	C
C. indica	12		
C. starrii	12		
C. aculeata	13		
C. pitschmannii	13		
C. geitleri	14		
C. pinicola	14		
C. hindakii	14		
C. terricola	14		
C. monoica	14		
C. noctigama	14		
C. brannonii	15		
C. texensis	15		
C. chlamydogama		II	C
C. moewusii		II	C
C. dorsoventralis		II	
C. sphaerella		II	
C. rosae		II	
C. pulsatilla		II	
C. reginae		II	
C. fimbriata		II	

Table 3. Grouping of algae in the order Volvocales on the basis of cell walls and their degrading enzyme specificities.

Family	Species	Roberts *et al.* (1982)	Matsuda *et al.* (1987a)
Astrephomenaceae	*Astrephomene gubernaculifera*		A
	A. perforata		A
Volvocaceae	*Gonium multicoccum*		A
	G. octonarium		A
	G. pectorale		A
	G. quadratum		A
	G. sacculiferum		A
	G. sociale		A
	Pandorina morum	I	B
	Volvulina steinii		B
	Eudorina elegans	I	B
	Pleodorina californica		B
	Volvox aureus	I	
	V. carteri		B
	Stephanosphaera pluvialis		C
Phacotaceae	*Dysmorphococcus globosus*		B
	Phacotus lenticularis		C
	Pteromonas angulosa		C
Chlamydomonadaceae	*Haemotococcus lacustris*	II	C
	Carteria crucifera	II	C
	C. eugametos	II	
	Chlorogonium elongatum	II	C
	C. euchlorum	II	
	Polytoma uvella	II	C
	Lobomonas piriformis	IV	C
Spondylomoraceae	*Pyrobotrys casinoensis*		C
Polyblepharidaceae	*Dunaliella salina*		C
	Pedinomonas minor		C

crystal structure (class I). Interestingly, the class I structure is typical of some colonial volvocacean algae, *Pandorina*, *Eudorina* and *Volvox* (Table 3). *C. asymmetrica* belongs to class III, while *Lobomonas piriformis* has the class IV structure. That *C. reinhardtii* and *Volvox* are more closely related than *C. eugametos* is also indicated by biochemical and morphological analyses of the cell walls (Roberts 1974; Musgrave *et al.* 1983; Goodenough and Heuser 1985; Adair *et al.* 1987). In addition, a recent hybridization experiment by Adair *et al.* (1987) showed that hybrid walls are obtained when a chaotropic salt-soluble extract from *Volvox carteri* is incubated with a salt-insoluble fraction (*i.e.* the inner wall layer) from *C. reinhardtii* and *vice versa*, whereas no interspecific assembly occurs between *C. reinhardtii* and *C. eugametos*.

In order to further investigate the relationships between the cell wall glycoproteins of *Chlamydomonas* and between those of related algae, Roberts *et al.* (1985b) raised a rabbit antiserum to one (termed B2; 270 kDa) of the major glycopolypeptides of the *C. reinhardtii* cell wall. Western blotting analysis showed that the antiserum cross-reacts with the cell wall of *C. angulosa* (class I) and *Lobomonas* (class IV), but does not

bind to the glycoproteins from the cell walls of *C. moewusii* (class II) or *C. asymmetrica* (class III). An indirect immunofluorescence study showed that the cell walls of *Volvox aureus*, *Eudorina elegans*, *Pandorina morum* and *Gonium pectorale* all fluoresce brightly with the antiserum (ROBERTS *et al.* 1985b). MATSUDA *et al.* (1987a) extended the immunofluorescence study using more than 40 species belonging to six families in the Volvocales (Tables 2 and 3). Among 15 species of *Chlamydomonas*, *C. globosa*, *C. incerta*, *C. smithii*, *C. cribrum*, *C. komma* and *C. angulosa* are labeled strongly with anti-glycopeptide B2, while others show no or weak cross-reactivity with the antibody. The antibody also cross-reacts strongly with the cell walls of a unicellular alga belonging to the Phacotaceae (*Dysmorphococcus*) and many colonial algae belonging to the Volvocaceae (*Gonium*, *Pandorina*, *Volvulina*, *Eudorina*, *Pleodorina*, *Volvox*) and the Astrephomenaceae (*Astrephomene*). On the other hand, unicellular algae, *Haematococcus*, *Phacotus*, *Carteria*, *Pteromonas*, *Polytoma*, *Chlorogonium* and *Lobomonas*, and colonial algae, *Pyrobotrys* and *Stephanosphaera* all have cell walls that do not react with the antibody (MATSUDA *et al.* 1987a). A similar pattern of labeling was obtained when a polyclonal antiserum raised against deglycosylated 2BII fraction, which recognizes only the protein core of 2BII glycoprotein molecules (ROBERTS *et al.* 1985b), was used except that the cell wall of *C. angulosa* cross-reacted with the anti-deglycosylated 2BII much more weakly than the anti-glycopeptide B2 (MATSUDA *et al.* unpublished data).

2. *Hatching enzyme as a taxonomic marker*

Hatching enzyme activity can be demonstrated in all species of *Chlamydomonas* which can be grown in synchronous cultures. According to the group-specific action of hatching enzymes, SCHLÖSSER (1976, 1984) has classified 65 strains of *Chlamydomonas* into 15 groups (Table 2). For example, *C. reinhardtii* belongs to group 1; a crude solution of hatching enzyme from this alga can dissolve the sporangial walls from all algae of group 1 (*C. globosa*, *C. incerta* and *C. smithii*), and *vice versa*, but cannot act on those from algae of other groups. In most cases, the mutual lytic action is restricted to within the group, but there are some exceptions: hatching enzymes from group 2 act non-reciprocally on all strains of group 1, and those from group 3 act on group 4 (SCHLÖSSER 1976).

In *Volvox*, mature autocolonies escape from the parental spheroid through the enzymatic rupture of the peripheral somatic cell layer (JAENICKE and WAFFENSCHMIDT 1979, 1981). JAENICKE and WAFFENSCHMIDT (1981) have purified the hatching enzyme from *V. carteri* and characterized it as a protease with tryptic properties. The enzyme has limited species specificity (*V. aureus* is sensitive, *V. africanus* is less sensitive, and *V. dissipatrix* and *V. gigas* are not sensitive), and does not act on the cell wall of *C. reinhardtii* wall.

3. *Lytic enzyme as a taxonomic marker*

In conjunction with the analysis of antibody binding (see above), MATSUDA *et al.* (1987a) have analyzed the sensitivity of cell walls of a variety of members of the Volvocales to lytic enzyme of *C. reinhardtii*. In the genus *Chlamydomonas*, the lytic enzyme acts only on the cell walls of all algae of group 1 (see also SCHLÖSSER *et al.* 1976), *C. komma* which belongs to group 2 according to SCHLÖSSER (1976), and *C. cribrum* which belongs to class I according to ROBERTS *et al.* (1982) (Table 2). Among other genera tested, the cell walls of *Gonium* and *Astrephomene* are sensitive to the *C. reinhardtii* lytic enzyme: their colonial structures are broken into individual cells by exposure to the enzyme, and protoplasts are then formed. Formalin-fixed colonies are also sensitive to the enzyme, suggesting a direct action of lytic enzyme on the cell wall lysis. Hatching enzyme of *C. reinhardtii* appears to be unable to break up the colonial structures of *Gonium* and *Astrephomene* (MATSUDA *et al.* 1987a).

If the sensitivity to lytic enzyme is combined with the labeling with anti-cell wall glycopeptide B2, the algae in the Volvocales can be divided into three classes (Tables 2 and 3): class A organisms (six species of *Chlamydomonas* including *C. reinhardtii* (*C. reinhardtii* group), six species of *Gonium* and two species of *Astrephomene*) whose cell walls are sensitive to the enzyme and show a strong cross-reactivity with the antibody, class B organisms (*C. angulosa. Dysmorphococcus*, *Pandorina*, *Eudorina*, *Volvulina*, *Pleodorina* and *Volvox*) whose cell walls are resistant to the enzyme, but show a strong cross-reactivity with the antibody, and class C organisms (many other species of *Chlamydomonas*, *Carteria*, *Chlorogonium*, *Polytoma*, *Haematococcus*, *Lobomonas*, *Phacotus*, *Pteromonas*, *Stephanosphaera* and *Pyrobotrys*) whose cell walls are resistant to the enzyme and show no or weak cross-reactivity with the antibody. Since lytic enzyme digests the inner wall layer of the *Chlamydomonas* cell wall while antibody will recognize the outer layers (see above), the cell walls of the class A organisms might be similar in chemical composition and arrangement of components to those of *C. reinhardtii* itself.

Taken together, all lines of evidence suggest that the members in the genus *Chlamydomonas* are composed of phylogenically diverse groups. For example, *C. reinhardtii* which belongs to group 1 appears to be closely related to other members in the same group and group 2, but distantly related to many other algae (*e.g. C. eugametos*) in other groups. Moreover, *C. reinhardtii* and its relatives (the *C. reinhardtii* group) are on the evolutionary line leading to the multicellular members of the Volvocales (Roberts *et al.* 1982; Matsuda *et al.* 1987a). Matsuda *et al.* (1987a) have proposed that there is an evolutionary sequence from a *C. reinhardtii*-like ancestor to the colonial algae, *Gonium* and *Astrephomene* (Fig. 5). There might also be a line of evolution from an ancestor of the *C. reinhardtii* to the volvocacean algae (except

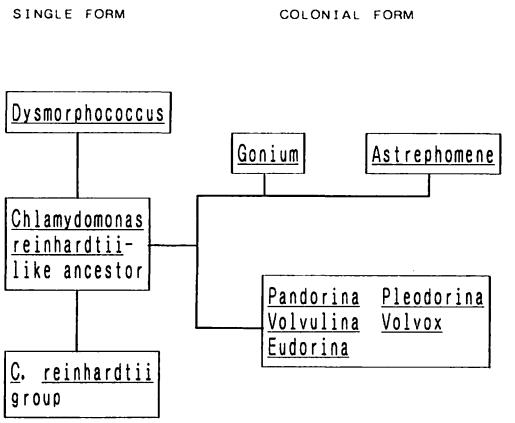

Fig. 5. Schematic representation of evolutionary lines from a *C. reinhardtii*-like ancestor to unicellular and colonial algae in the Volvocales.

Stephanosphaera) because of the similarities of the structure and chemical composition of their cell walls (Fig. 5). These phylogenic relationships agree well with those suggested from microscopically visible features (Fulton 1978a; Nozaki 1986). The pattern of cell cleavage in *Gonium* (specifically, *G. pectorale* and *G. octonarium*) and *Astrephomene* is a parallel-type, whereas that in *Pandorina*, *Volvulina*, *Eudorina*, *Pleodorina* and *Volvox*, which undergo inversion during the colony formation, is a rotational-type (Pocock 1953; Stein 1958a, b; Gerisch 1959; Goldstein 1964; Starr 1969; Fulton 1978b; Nozaki 1983, 1986). *Stephanosphaera* shows a radial-type cleavage (Hieronymus 1884). Furthermore, every cell in a colony of *Gonium* and *Astrephomene* is surrounded by the inner and outer layers of cell wall, whereas in other volvocacean algae, the outer layers do not cover over the individual cells but surround the colonial surface as an extracellular matrix (Fulton 1978a; Nozaki 1986; Nozaki *et al.* 1987).

The cell walls and their degrading enzymes will constitute very important taxonomic keys in future studies to investigate more detailed phylogenic relationships between chlamydomonads and other members of the Volvocales.

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Note Added in Proof: Recently, LEMIEUX *et al.* (BioSystems 1985, **18**: 293–298) and JUPE *et al.* (BioSystems 1988, **21**: 223–230) have analyzed the chloroplast DNA and nuclear rRNA genes of *Chlamydomonas reinhardtii, C. moewusii* and *C. eugametos* for the potential application to the systematics of *Chlamydomonas,* and suggested a large phylogenetic distance between *C. reinhardtii* and *C. moewusii* or *C. eugametos* (the latter two strains are interfertile and similar in molecular comparisons).

松田吉弘：クラミドモナスの細胞壁とその溶解酵素

オオヒゲマワリ目は，クラミドモナスのような単細胞性藻からオオヒゲマワリのような群体性藻まで多彩な藻類で構成されているが，これらの細胞壁や外被は，いずれも糖蛋白質より成り，高等植物や他の藻類でみられるポリグルカンを殆ど持たない。細胞壁の構造，生化学，構成成分の合成と分泌，人為的分解と再構成，さらに突然変異については，とくに *Chlamydomonas reinhardtii* を材料として研究が進められており，ここでは，まずこれらの最近までの知見を概説した。また，*C. reinhardtii* では，プラス型，マイナス型両配偶子が接合する時に分泌される細胞壁溶解酵素（リティックエンザイム）と無性的増殖時に母細胞壁を破って娘細胞が放出されるために必要な酵素（ハッチングエンザイム）の両者が，最近単離精製された。これらのプロテアーゼ性細胞壁溶解酵素の分子性状，細胞壁の分解様式，細胞内存在形態および存在場所，分泌シグナルなどをまとめて論議した。これまで *Chlamydomonas* の種レベルでの分類は，主として光学顕微鏡下のごく僅かな形態的特徴の違いに基いたものであり，問題が多い。そこで，最後に，*Chlamydomonas* 属内での種のグルーピングや，オオヒゲマワリ目内での単細胞性藻と群体性藻の間での系統的類縁関係を論議するためになされた，細胞壁と二種類の溶解酵素の特異性を利用したいくつかの研究を紹介した。（657 神戸市灘区六甲台1-1　神戸大学理学部生物学教室）