

# Topography of Cell Wall Lytic Enzyme in *Chlamydomonas reinhardtii*: Form and Location of the Stored Enzyme in Vegetative Cell and Gamete

Yoshihiro Matsuda, Tatsuaki Saito, Tetsuya Yamaguchi, Machi Koseki, and Kazuko Hayashi

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

**Abstract.** *Chlamydomonas* lytic enzyme of the cell wall (gamete wall-autolysin) is responsible for shedding of cell walls during mating of opposite mating-type gametes. This paper reports some topographic aspects of lytic enzyme in cells. Both vegetative and gametic cells contain the same wall lytic enzyme. The purified enzyme is a glycoprotein with an apparent molecular mass of 67 kD by gel filtration and 62 kD by SDS PAGE, and is sensitive to metal ion chelators and SH-blocking agents. These properties are the same as those of the gamete wall-autolysin released into the medium by mating gametes. However, the storage form of the enzyme proves to be quite different between the two cell types. In vegetative cells, the lytic enzyme is found in an insoluble form in cell homogenates and activity is released into the soluble fraction only by sonicating the homogenates or freeze-thawing the cells, whereas gametes always yield lytic

activity in the soluble fractions of cell homogenates. When vegetative cells are starved for nitrogen, the storage form of enzyme shifts from its vegetative state to gametic state in parallel with the acquisition of mating ability. Adding nitrogen to gametes converts it to the vegetative state concurrently with the loss of mating ability. We also show that protoplasts obtained by treatment of vegetative cells or gametes with exogenously added enzyme have little activity of enzyme in the cell homogenates, suggesting that lytic enzyme is stored outside the plasmalemma. When the de-walled gametes or gametes of the wall-deficient mutant, *cw-15*, of opposite mating types are mixed together, they mate normally but the release of lytic enzyme into the medium is practically negligible. When the de-walled vegetative cells are incubated, the lytic enzyme is again accumulated in the cells after the wall regeneration is almost complete.

CELL wall lytic enzymes are responsible in the life cycle of plant cells for wall extension, zoospore hatching, and partial or total dissolution of the cell wall during conjugation (32, 35). In the unicellular, biflagellated alga *Chlamydomonas reinhardtii*, shedding of the gametic cell wall takes place during mating as a necessary prelude to cell fusion (2). This event is caused by a cell wall lytic enzyme, referred to as gamete wall-autolysin (31), which is induced by the signal of flagellar agglutination. Gametes slip away from the walls by breaking off their apical portion with lytic enzyme and concurrently excrete the enzyme into the medium (recently reviewed in references 34 and 37). Using this mating medium as the starting material, we have previously purified and identified the gamete wall-autolysin as a single glycopolypeptide of 62 kD by SDS PAGE (23) and then characterized it as a zinc-containing metalloprotease (20). Furthermore, it has been reported recently (4, 10, 20) that the sodium perchlorate-insoluble (framework) portion (1) of the multilayered structure (26) of the *Chlamydomonas* cell wall is the target of the lytic enzyme.

The pioneering work on the *Chlamydomonas* lytic factor

by Claes (2) has shown that lytic activity found in the mating medium can also be detected in vegetative and gametic cells. However, the identity of the gamete wall-autolysin with the vegetative and gametic enzymes remains to be clarified. In addition, the form and location of the stored enzyme in these two cell types are still unknown. We present here some topographic aspects of cell wall lytic enzyme. We demonstrate by purification and characterization studies that the same enzyme molecules as gamete wall-autolysin exist both in vegetative and gametic cells. However, their storage forms in the cells appear to be quite different between the two cell types. Our results indicate that the vegetative enzyme is stored in an insoluble and inactive form, whereas the gametic enzyme is, at least partly, in a soluble and active form. Of particular importance is our finding that the storage form shifts back and forth between the two according to the gametic differentiation and dedifferentiation of the cell. We also suggest that the lytic enzyme is localized outside the plasmalemma and excreted into the medium during mating of gametes.

## Materials and Methods

### Cell Strains and Culture Conditions

The wild-type strain 137c, mating-type plus ( $mt^+$ )<sup>1</sup> and minus ( $mt^-$ ), of *Chlamydomonas reinhardtii* and the cell wall-less mutant strains,  $cw-2$   $mt^+$ ,  $cw-3$   $mt^+$ ,  $cw-8$   $mt^+$ ,  $cw-10$   $mt^-$ ,  $cw-15$   $mt^+$ , and  $cw-15$   $mt^-$  (9) were used. All the mutant strains were obtained from the *Chlamydomonas* Genetics Center, Department of Botany at Duke University (Durham, NC), with the exception of  $cw-15$ , obtained from the Cambridge Algae Collection. Vegetative cells were grown in constant light in liquid minimal medium for ~40 h (22). Gametes were obtained by flooding 6-d-old cultures on 1.5% agar plates containing minimal medium with nitrogen-free induction medium at  $5 \times 10^7$  cells/ml and by incubating for 2–3 h (29). In certain experiments, vegetative cells in liquid cultures were harvested by centrifugation at 700 g for 2 min, washed twice in induction medium, and resuspended at  $5 \times 10^6$  cells/ml in the medium to induce gametes (22). To initiate dedifferentiation, gamete cultures at  $5 \times 10^7$  cells/ml were transferred to 9 vol of minimal medium and incubated. Agglutininability and fusing ability (mating efficiency) of cells were determined by mixing them with tester gametes of the opposite mating-type in equal numbers as described previously (21, 22).

### Preparation of Cell Homogenates and Their Fractionation

Vegetative or gametic cells were harvested by centrifugation, resuspended in ice cold 10 mM Tris-acetate, pH 7.5, at  $2-5 \times 10^8$  cells/ml, and homogenized by passing through a French pressure cell at 400 kg/cm<sup>2</sup> at 4°C. Complete breakage of cells by French press was regularly monitored by phase-contrast microscopy. In some experiments, the pelleted cells were frozen at -80°C overnight and thawed before homogenization. Fractionation of homogenates was carried out either by differential centrifugation ranging from 1,000 to 100,000 g or by density gradient centrifugation using Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) at 4°C. Homogenates were centrifuged at 8,000 g for 15 min to remove large cell debris, and the supernatant was added with Percoll and sucrose to give a final concentration of 15% and 0.25 M, respectively. After centrifugation at 50,000 g for 30 min with a Hitachi RP50T2 rotor, fractions of 1 ml were collected. The density was determined from refractive index. Sonication of homogenates before and after fractionation was performed using a Tomy Seiko Ultrasonic Vibrator (model UR-200P) at 4°C for 1 min at designated powers.

### Methods of Lytic Enzyme Purification

Gamete wall-autolysin was purified from the mating medium by the procedure modified from that described previously (20, 23). The  $mt^+$  and  $mt^-$  gametes were mixed in equal numbers and allowed to mate for 10 min at 25°C. The mating mixture (1 liter) was then added with one-fourth volume of ice cold 50 mM Tris-acetate, pH 7.5, and 1 M NaCl (5× solution A), and centrifuged at 8,000 g for 10 min at 4°C. Ammonium sulfate was added to the cell-free supernatant to 60% saturation for 30 min on ice. After centrifuging at 13,000 g for 20 min, the pellets were resuspended in 50 ml of 10 mM Tris-acetate, pH 7.5, and 200 mM NaCl (solution A), dialyzed overnight at 4°C against solution A, and centrifuged at 100,000 g for 30 min. The supernatant was applied to concanavalin A (Con A)-Sephacryl 4B gel (Pharmacia Fine Chemicals; 2.2 × 12 cm column) at a flow rate of 25 ml/h. After extensive washing of the column with solution A, enzyme was eluted with 0.1 M methyl- $\alpha$ -D-mannoside in solution A. Fractions containing lytic activity were pooled, concentrated by collodion bag (Sartorius) to ~1 ml, and fractionated by gel filtration over Sephacryl S-200 Superfine (Pharmacia Fine Chemicals) packed in a 2.2 × 30 cm column at a flow rate of 30 ml/h. The most active fractions were pooled, bound to a 2-ml bed of hydroxyapatite, and eluted with 50 mM sodium phosphate in solution A. The fractions containing lytic activity were pooled, concentrated, and rechromatographed through a second gel filtration column (2.2 × 60 cm) at a flow rate of 10 ml/h. The purified enzyme thus obtained was concentrated by collodion bag and stored at -80°C.

Vegetative and gametic enzymes were purified in the following ways. Harvested  $mt^+$  cells were frozen, thawed, resuspended in 10 mM Tris-acetate at  $5 \times 10^8$  cells/ml, and broken by French press as described above.

1. Abbreviations used in this paper:  $mt^+$ , mating-type plus;  $mt^-$ , mating-type minus.

After a 60-min centrifugation at 100,000 g, the supernatant (~30 ml) was added with 60 ml of DEAE-cellulose (Whatman Inc., Clifton, NJ; DE 52) which had been equilibrated with 10 mM Tris-acetate. The mixture was stirred gently for 30 min at 4°C, filtered in a funnel, and the DEAE-cellulose was washed twice with the buffer. The filtrates, in which most of lytic enzyme was recovered (see Results), were combined and added with one-fourth volume of 5× solution A. Subsequently, Con A affinity chromatography, gel filtration, hydroxyapatite chromatography, and the second gel filtration were carried out by the same procedures as those described for the purification of gamete wall-autolysin.

### Enzyme Assay

The activity of cell wall lytic enzyme was estimated with the glutaraldehyde-fixed zoosporangia as substrates by the method described previously (23, 36). The reaction mixtures contained 10 mM Tris-acetate, pH 7.5, 0.5 mg/ml BSA,  $2.5 \times 10^8$  glutaraldehyde-fixed zoosporangia, and 0–240  $\mu$ l enzyme solution in a total volume of 250  $\mu$ l. The reaction was run at 35°C for 30 min and stopped by addition of EDTA to give a final concentration of 20 mM. After stirring vigorously in a Vortex mixer for 15 s, the extent of zoospore liberation was determined by phase-contrast microscopy. One unit of activity is defined as the amount of enzyme which liberates daughter cells at the 50% level.

Acid phosphatase activity was determined by the method of Loppes and Matagne (16) modified from MacIntyre (17) using 1-naphthylphosphate as a substrate. Cell homogenates were centrifuged at 100,000 g for 60 min and the supernatants (soluble fractions) were used for enzyme assay.

### SDS PAGE

SDS PAGE was carried out in the discontinuous buffer system of Neville (25) using 7.5–15% linear acrylamide gradients with 3.5% acrylamide stacker. Sample preparation and gel stain (silver) were performed as described previously (23, 29).

### Protoplast Formation

Walled vegetative cells or gametes ( $\sim 5 \times 10^6$  cells/ml) were incubated with 20–50 U/ml crude lytic enzyme and 0.5 mg/ml BSA at 25°C for 30 min (23). Protoplast formation was monitored by the heating method as described previously (22) and was usually above 95%. For the reformation of cell walls after enzyme treatment of vegetative cells, the lytic enzyme was washed away by centrifugation, and the cells were incubated in minimal medium supplemented with 1 g/liter sodium acetate at  $5 \times 10^6$  cells/ml.

### Inhibitors

Phenylmethylsulfonyl fluoride (PMSF) was dissolved in isopropanol at 40 mM, *p*-chloromercuribenzoic acid in 40 mM NaOH at 50 mM, and 1,10-phenanthroline in 95% ethanol at 100 mM (6). HgCl<sub>2</sub>, iodoacetate, and cycloheximide were dissolved in distilled water at 100 mM, 500 mM, and 1 mg/ml, respectively. EDTA was dissolved in 10 mM Tris-HCl, pH 7.0, at 200 mM. The specified amounts of these stock solutions were added to the enzyme solutions or cell suspensions.

Table I. Activity of Cell Wall Lytic Enzyme in Cell Homogenates and Mating Medium

Source	Lytic activity		
	Homogenate		
	Unfrozen cell	Frozen cell	Medium
	<i>U/10<sup>6</sup> cells</i>	<i>U/10<sup>6</sup> cells</i>	<i>U/10<sup>6</sup> cells</i>
Vegetative cell			
<i>mt<sup>+</sup></i>	<2	460 ± 65	—
<i>mt<sup>-</sup></i>	<2	400 ± 80	—
Gamete			
<i>mt<sup>+</sup></i>	500 ± 60	400 ± 80	—
<i>mt<sup>-</sup></i>	370 ± 80	430 ± 60	—
Gamete mixture	—	—	2,560 ± 180

Values are expressed as mean ± SD from three separate experiments.

**Table II. Activities of Lytic Enzyme and Acid Phosphatase in the Soluble Fraction Obtained before and after Homogenization of *mt*<sup>+</sup> Cells**

Cell	Lytic enzyme		Acid phosphatase	
	Before FP	After FP	Before FP	After FP
	U/10 <sup>9</sup> cells		μmol naphthol/10 <sup>9</sup> cells per h	
Vegetative				
Unfrozen	<2	<2	0	12.1
Frozen	<2	400	11.7	12.7
Gametic				
Unfrozen	<2	500	0	5.8
Frozen	<2	500	4.1	5.4

Unfrozen and frozen cells were resuspended in 10 mM Tris-acetate buffer. Half the amount of cells was centrifuged at 100,000 g for 60 min and the soluble fraction was saved. The other half was passed through the French press (FP); the homogenates were then centrifuged and the soluble fraction was obtained.

## Results

### Lytic Activity in Homogenates and the Soluble Fractions of Vegetative Cells and Gametes

The wild-type *C. reinhardtii* cells were homogenized in a French pressure cell, the homogenate was spun at 100,000 g for 1 h, and the supernatant termed soluble fraction was saved. In these experiments, half the amount of cells was frozen and thawed before homogenization. Tables I and II summarize the lytic activity found in the homogenates and the soluble fractions obtained from unfrozen and frozen cells. We first noted in vegetative cells that there is a distinct difference in the activity between unfrozen and frozen cells. Neither the homogenate (Table I) nor the soluble fraction prepared from unfrozen cells (Table II) had any detectable activity of lytic enzyme, whereas both preparations from frozen cells yielded practically the same level of activity (~400 U/10<sup>9</sup> cells). Since unfrozen cells as well as frozen cells were observed by phase-contrast microscopy to be disrupted completely after passing through the French press, the lack of activity in the former preparation was not due to nonbreakage of cells. Furthermore, we found that the activity of the soluble acid phosphatase, which is located in the vacuoles in *Chlamydomonas* (19), was equally found in the soluble fractions of homogenates from unfrozen and frozen cells (Table II). These results indicate that the lytic enzyme in vegetative cells is released into the soluble fraction by freeze-thawing the cells before homogenization.

Different from vegetative cells, both unfrozen and frozen cells of gametic origin yielded the same level of lytic activity in the homogenates (Table I) and in the soluble fractions (Table II). However, it was noticed that homogenization by the French press is required to release the gametic enzyme into the soluble fraction since no activity was found in the soluble fraction of frozen cells without homogenization (Table II). This was a sharp contrast to the soluble acid phosphatase in which the activity could be released into the soluble fraction only by freeze-thawing the cells (Table II).

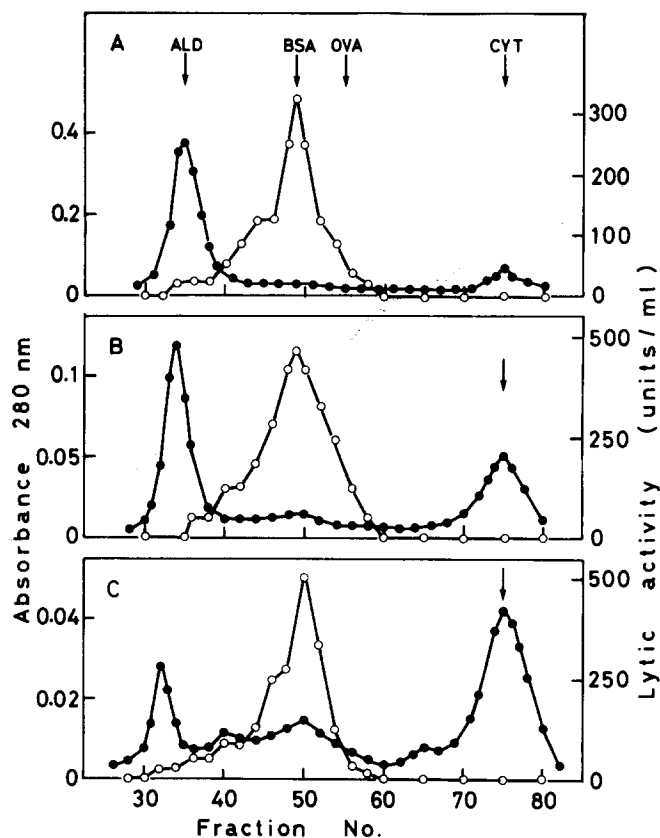
Although the *mt*<sup>+</sup> and *mt*<sup>-</sup> vegetative cells and gametes appeared to have the same amount of lytic activities, this amount was significantly lower (~20% level) than that of the

lytic activity (~2,500 U/10<sup>9</sup> mixed gametes) present in the medium of mating gametes (Table I). We found in the following experiments that the difference is due to the presence of some inhibitor(s) of lytic activity in the soluble fraction of cell homogenates.

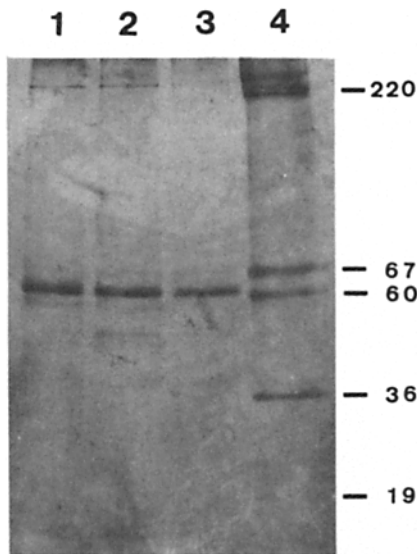
### Purification and Characterization of Lytic Enzyme in Cells

To see whether the lytic enzymes found in vegetative cells and gametes are identical, they were purified using the soluble fractions of homogenates from frozen cells, and their characters were compared with each other or with gamete wall-autolysin which had been purified from the mating medium (23).

The first batch use of DEAE-cellulose at low salt condition proved to be valuable for enzyme purification since a large amount of protein impurities and some inhibitor(s) of lytic activity were bound to DEAE-cellulose. The vegetative and gametic enzymes, however, were not absorbed at all,



**Figure 1.** Purification of lytic enzymes from vegetative cells, gametes, and mating medium by gel filtration. After Con A affinity chromatography and concentration, 1 ml of (A) the vegetative enzyme (3,000 U), (B) the gametic enzyme (5,200 U), and (C) the gamete wall-autolysin (5,000 U) were fractionated by Sephacryl S-200 chromatography. Before fractionation, the samples were mixed with 0.25 ml (0.5 mg) of cytochrome C used as a marker. Fractions of 1 ml were collected and analyzed for the absorbance at 280 nm (solid circles) and lytic activity (open circles). Standard proteins used for the determination of the molecular mass of lytic enzymes were aldolase (ALD; 155 kD), bovine serum albumin (BSA, 66 kD), ovalbumin (OVA, 45 kD), and cytochrome C (CYT, 12.5 kD). Arrows indicate the peak positions of standard proteins.



**Figure 2.** SDS PAGE of purified lytic enzymes. After a second gel filtration, the activity peak fraction ( $\sim 100$  U) was analyzed by SDS PAGE (7.5–15% acrylamide gradient gel) and silver staining. Lane 1, Vegetative enzyme; lane 2, gametic enzyme; lane 3, gamete wall–autolysin; lane 4, marker proteins: ferritin half unit (220 kD), bovine serum albumin (67 kD), catalase (60 kD), lactate dehydrogenase (36 kD), and ferritin (19 kD).

resulting in elimination of  $\sim 90\%$  of the total protein after this purification step. In addition, the elimination of inhibitor(s) by DEAE–cellulose binding resulted in a three- to fivefold increase in total activity. There was no indication of total activity increase in the mating medium by the DEAE treatment. Similar to the gamete wall–autolysin (23), both enzymes were then bound specifically to a Con A–Sephrose column (the flow-through did not contain any lytic activities) and eluted with 0.1 M methyl- $\alpha$ -D-mannoside, indicating that these enzymes have mannose and/or glucose residues.

Further purification of the enzymes was achieved by gel filtration using Sephacryl S-200 (Fig. 1). Both vegetative (Fig. 1 A) and gametic enzymes (Fig. 1 B) eluted in one peak of activity at the same position with an apparent molecular mass of  $67 \pm 2$  kD (mean value with SD in three separate analyses). Furthermore, this value was quite similar to that of gamete wall–autolysin ( $65 \pm 4$  kD in three separate analyses) (Fig. 1 C). After rechromatography of the vegetative and gametic enzymes on a second gel filtration column, the enzyme peak fractions were visible after SDS PAGE and silver staining as a single 62-kD polypeptide accompanied by two to three faint bands around this main band (Fig. 2, lanes 1 and 2). The 62-kD polypeptide corresponded exactly to the gamete wall–autolysin on SDS PAGE (lane 3). This molecule is therefore considered to be the vegetative and gametic enzymes.

Table III lists the effects of potent inhibitors of serine, thiol, and metalloproteases on the purified vegetative and gametic enzymes. Chelators of the metal ions (EDTA, 1,10-phenanthroline) and SH-blocking agents (*p*-chloromercuribenzoic acid, iodoacetate,  $\text{HgCl}_2$ ) have previously been shown to inactivate the gamete wall–autolysin (23) and did also inactivate the two cellular enzymes. By contrast, PMSF, which is a potent inhibitor of serine protease and ineffective

**Table III.** Effects of Inhibitors on Lytic Activity of Enzyme Purified from Vegetative Cells and Gametes

Inhibitor	Concentration	Percent inhibition	
		Vegetative enzyme	Gametic enzyme
	<i>mM</i>		
PMSF	1.0	0	0
EDTA	0.02	12	8
	0.05	100	100
$\text{HgCl}_2$	0.2	6	100
	0.5	100	100
Iodoacetate	4	67	68
	5	100	100
<i>p</i> -Chloromercuribenzoic acid	2.5	10	36
	5	100	100
1,10-Phenanthroline	0.05	18	100
	0.1	59	100

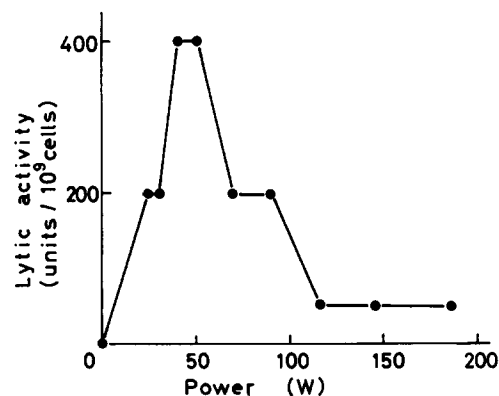
Purified enzymes after the second gel filtration were used. Inhibitors were added to reaction mixtures containing 0.25–0.5 U of enzyme activity, and reaction was started by adding zoosporangia as substrate.

against gamete wall–autolysin (23), did not abolish the vegetative and gametic enzymes.

From all the above results we conclude that both vegetative cells and gametes contain the same cell wall lytic enzyme as gamete wall–autolysin.

#### Solubilization of Lytic Enzyme from Homogenates of Unfrozen Vegetative Cells

Although vegetative cells had no detectable lytic activity unless they were frozen before homogenization (see above), sonication was found to be effective to release the lytic enzyme from the homogenate of unfrozen vegetative cells (Fig. 3). At an appropriate power setting ( $\sim 50$  W), the sonicated homogenates had the same amount of lytic activity as the homogenates from frozen cells. Furthermore, almost all the activity was observed in the soluble fraction after sonication. To see where the lytic enzyme is concealed, the homogenate from unfrozen cells was first fractionated by differential centrifugation and then each fraction was sonicated to solubilize



**Figure 3.** Sonication of homogenates from unfrozen vegetative cells. Unfrozen vegetative cells were homogenized completely by French press at a density of  $5 \times 10^8$  cells/ml. Aliquots of 2 ml were sonicated at different power settings shown on the abscissa for 1 min at 4°C, and lytic activities were determined.

**Table IV. Distribution of Lytic Activity after Fractionation of Homogenate from Unfrozen Vegetative Cells by Differential Centrifugation**

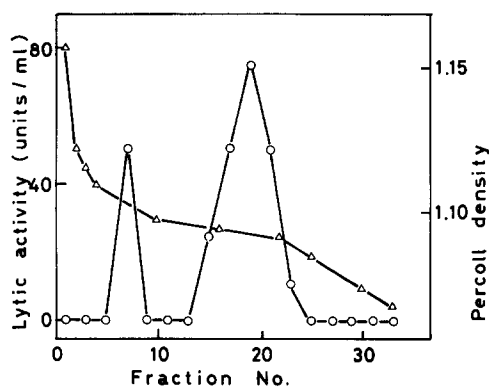
Fraction	Lytic activity
	%
1,000 g pellet	6.8
8,000 g pellet	6.8
15,000 g pellet	56.4
30,000 g pellet	17.1
100,000 g pellet	8.5
100,000 g supernatant (soluble fraction)	4.3

Homogenate was prepared from the *mt*<sup>+</sup> vegetative cells without prefreezing. Centrifugations were carried out for 15 min with the exception of 100,000 g, done for 60 min. Pellets were resuspended in 10 mM Tris-acetate buffer and sonicated at 50 W for 1 min to solubilize the enzyme.

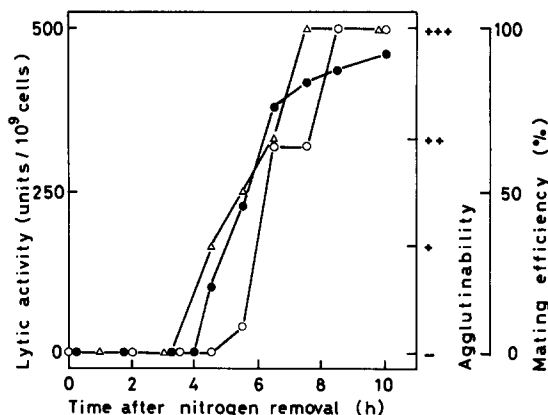
the enzyme. Table IV shows that the 15,000 g and 30,000 g pellets contain a considerable amount of enzyme in an inactive form. Fig. 4 shows the distribution pattern of the inactive enzyme after Percoll density gradient centrifugation. Cell homogenate was spun at 8,000 g to remove the large cell fragments, and the supernatant was mixed with Percoll, centrifuged at 50,000 g, and fractionated. By sonicating each fraction, lytic activity was detectable as two peaks at a density of 1.085 and 1.100, respectively. These two activity peaks were reproducible in repeated experiments although their relative activity ratio was changed.

#### Changes in Enzymatic Behavior during Gamete Differentiation and Dedifferentiation

The above results clearly indicate that there is a distinct difference between the vegetative cells and the gametes with respect to lytic enzyme behavior. In *C. reinhardtii*, differentiation of vegetative cells to gametes is a reversible process that can be controlled experimentally (11, 28). Withholding a nitrogen source induces gametogenesis, and adding it back converts gamete to vegetative cell. We therefore used the



**Figure 4.** Percoll density gradient fractionation of homogenate from unfrozen vegetative cells. Cells were broken, centrifuged at 8,000 g for 15 min to remove the large cell fragments, and the supernatant (20 ml) was mixed with Percoll and sucrose to give a final concentration of 15% and 0.25 M. After centrifugation at 50,000 g for 30 min, fractions of 1 ml were collected, and analyzed for the density (open triangles) and lytic activity (open circles) after sonicating at 50 W for 1 min.



**Figure 5.** Changes in enzymatic behavior during differentiation of gametes. Vegetative cells of *mt*<sup>+</sup> in nonsynchronized liquid culture were transferred to nitrogen-free medium at a density of  $5 \times 10^6$  cells/ml. At the times indicated, 1-ml portions of cells were taken and mixed with the tester gametes of *mt*<sup>-</sup> in equal numbers to determine their agglutinability (open triangles) and mating efficiency (solid circles). At the same time, 100-ml portions were harvested by centrifugation at 4°C, resuspended in 2.5 ml of ice cold 10 mM Tris-acetate, pH 7.5, and the suspensions were passed through the French press. Lytic activity (open circles) was assayed by use of the homogenates.

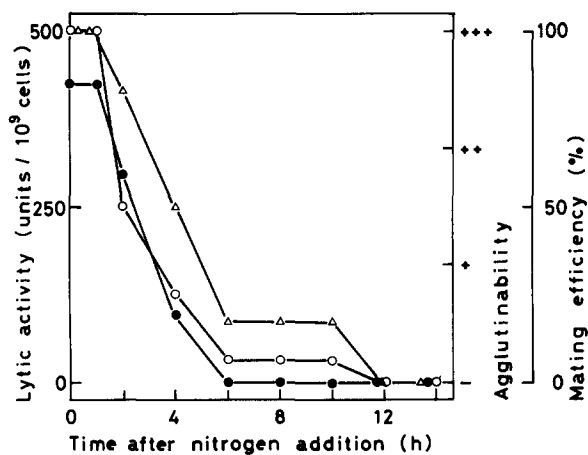
differentiation and dedifferentiation processes to examine how the lytic enzyme changes its behavior.

Since we can now distinguish between the two cell types by a simple determination of lytic activity containing in the homogenates from unfrozen cells (Table I), this assay method was used in the following experiments. Fig. 5 gives plots of lytic activity determined during gametogenesis that occurs when vegetative cells in nonsynchronized liquid culture are transferred to nitrogen-free medium. Lytic activity was not detectable during the first 4 h of gametic induction, then developed rapidly and reached the level of gametes after 8 h (Fig. 5). It is important that the transition of enzymatic behavior from its vegetative (i.e., inactive) to gametic (i.e., active) state occurred in parallel with the acquisition of agglutinability and fusing ability of cells (Fig. 5). During the gamete induction, the increase in cell number was negligible.

When fully differentiated gametes were returned to a nitrogen-containing medium, the lytic activity found in the homogenates from unfrozen cells decreased again in parallel with the loss of mating ability (Fig. 6). After a 14-h incubation, cells lost completely both activities (Table V). However, when the same cells were frozen, the homogenate had the same amount of lytic activity as gametic homogenate (Table V), indicating that the lytic enzyme returned completely to an inactive form characteristic of the vegetative cells. During the dedifferentiation of gametes, the increase in cell number was at most 20%. Table V also shows that cycloheximide, an inhibitor of protein synthesis, blocks the gamete dedifferentiation; that is, the loss of mating ability and the transition of lytic enzyme from the gametic to the vegetative form.

#### Localization of Lytic Enzyme

To determine whether the lytic enzyme is localized within the cytoplasmic membrane or outside, the cell walls of vegetative cells and gametes were removed by gamete



**Figure 6.** Changes in enzymatic behavior during dedifferentiation of gametes. Fully differentiated gametes of  $mt^+$  ( $5 \times 10^7$  cells/ml) were transferred to 9 vol of nitrogen-containing medium. At the times indicated, portions of cells were taken to determine their agglutinability (open triangles), mating efficiency (solid circles), and lytic activity (open circles) by the methods as described in the legend to Fig. 5.

wall-autolysin. The protoplasts thus obtained were harvested, washed to remove the digested cell walls and added enzyme, frozen, homogenized, and then the lytic activity was determined. The homogenates from vegetative cells and gametes after enzyme treatment had only 10% or less of lytic activity as compared to those from untreated cells (Table VI). These results indicate that the lytic enzyme is localized largely outside the cytoplasmic membrane. This was also indicated by use of the cell wall-deficient strains. All the vegetative cells of the cell wall-deficient strains,  $cw-2$ ,  $cw-3$ ,  $cw-8$ ,  $cw-10$ , and  $cw-15$  lacked any detectable lytic activity in their homogenates from frozen cells (Table VI). The level of lytic activity in gametes of  $cw-15$  was 3–5% that of the wild-type cells (Table VI).

To determine whether the lytic enzyme localized outside the plasmalemma is actually released into the medium during mating,  $mt^+$  and  $mt^-$  gametes of the wild-type cells, the wall-digested cells with enzyme or the  $cw-15$  mutant cells were mixed together, and kinetics of enzyme release into the medium was examined (Fig. 7). As Snell (33) has first reported, the release of lytic enzyme was complete within 5 min after opposite mating-type gametes were mixed together. In the mixture of wall-less cells treated with gamete wall-autolysin, agglutination and cell fusion occurred normally (mating efficiency, 87%), but the amount of lytic enzyme released was only 10% that of the walled cells (Fig. 7). This proportion was practically in agreement with that of lytic activity observed in the wall-digested cells before mixing (see Table VI). By mixing the walled gametes of opposite mating types, the lytic activity in cell homogenates was reduced to <4% level within 10 min.

Similarly, gametes of  $cw-15$  cells showed good agglutination and fusion (mating efficiency 76%), whereas the level of lytic enzyme released was <10% that of the wild-type cells (Fig. 7).

When protoplasts of vegetative cells are incubated in lytic enzyme-free medium, they reform new walls over a period of several hours (4, 27). Here we followed this process to see the correlation of lytic enzyme restoration with wall regener-

**Table V.** Loss of Mating Ability and Gametic Form of Lytic Enzyme during Dedifferentiation of Gametes and the Effect of Cycloheximide on Dedifferentiation

Treatment	Mating ability		Lytic activity	
	Agglutination	Fusion	Unfrozen cell	Frozen cell
–N	+++	61	250	ND
+ Cycloheximide	+++	26	500	ND
+N	–	0	<5	400
+ Cycloheximide	+++	18	400	400

Gametes ( $mt^+$ ) were incubated in nitrogen-free (–N) or -containing (+N) medium for 14 h. Cycloheximide (2  $\mu$ g/ml) was added at the beginning of incubation. ND, not determined.

ation. The development of lytic activity determined by use of frozen cells occurred after wall construction was almost complete (Fig. 8).

All the above results support the hypothesis that the lytic enzyme located within the periplasmic space or in the cell walls of gametes is excreted into the medium as the gamete wall-autolysin during mating.

## Discussion

### An Identical Lytic Enzyme Exists in Vegetative Cell and Gamete, but the Storage Form is Different

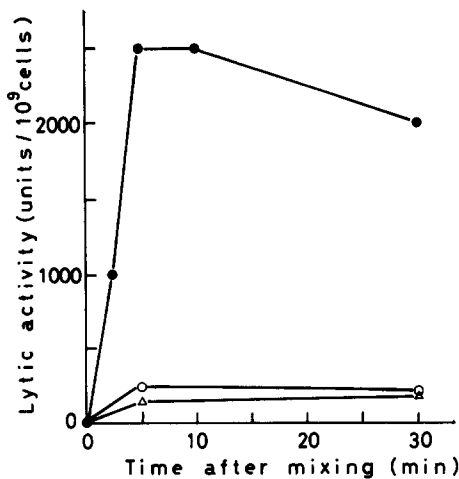
At the beginning of this study, we confirm Claes's finding (2) that the cell wall lytic activity is present both in vegetative cells and gametes of *C. reinhardtii*. Quantitative assays show that these two cell types and also both  $mt^+$  and  $mt^-$  cells have the same level of lytic activity (Table I). Purification studies show that both vegetative and gametic enzymes are a glycoprotein with an apparent molecular mass of 67 kD on gel filtration (Fig. 1) and 62 kD on SDS PAGE (Fig. 2). Moreover, these values are in agreement with those of gamete wall-autolysin which is excreted into the medium by mating gametes (Figs. 1 and 2 and reference 23). Further characterization of the purified enzymes by use of various protease inhibitors (Table III) shows that the cellular en-

**Table VI.** Lytic Activity in the Wild-type Cells Treated with Gamete Wall-Autolysin and in the Wall-less Mutant Cells

Strain	Culture*	Lytic activity†	
		Gamete wall-autolysin Untreated	Treated
		<i>U/10<sup>9</sup> cells</i>	<i>U/10<sup>9</sup> cells</i>
Wild-type $mt^+$	V	400	20
	G	450	40
$mt^-$	G	440	20
$cw-2$ $mt^+$	V	<5	–
$cw-3$ $mt^+$	V	<5	–
$cw-8$ $mt^+$	V	<5	–
$cw-10$ $mt^-$	V	<5	–
$cw-15$ $mt^+$	V	<5	–
	G	25	–
	V	<5	–
$mt^-$	V	<5	–
	G	15	–

\* V, vegetative culture; G, gametic culture.

† Activities were measured by use of homogenates from frozen cells.



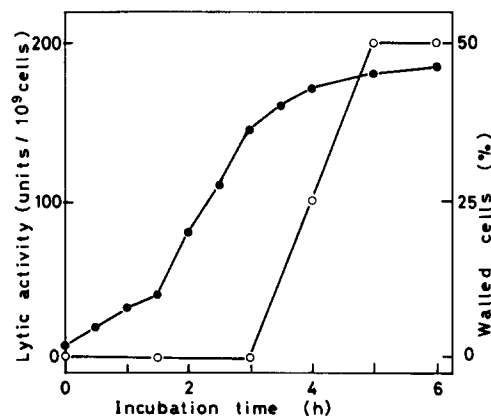
**Figure 7.** Release of lytic enzyme into the medium during mating of gametes. The wild-type cells (*solid circles*), their wall-digested cells (*open circles*), and the *cw-15* mutant cells (*open triangles*) of *mt<sup>+</sup>* and *mt<sup>-</sup>* were mixed together at  $5 \times 10^7$  cells/ml. At the times indicated, 1-ml portions were taken, centrifuged quickly, and the supernatant was assayed for lytic activity.

zymes are, like gamete wall-autolysin (23), sensitive to SH-blocking agents and metal ion chelators, but are insensitive to the inhibitor of serine protease. We therefore conclude that vegetative cells as well as gametes possess the same enzyme molecules as gamete wall-autolysin.

The present paper, however, demonstrates that there is a distinct difference between the vegetative and gametic enzymes with respect to their storage forms. We first note that freezing and homogenization are required to release the vegetative form of the enzyme into the soluble fraction, whereas the gametic form is released by homogenization alone (Table II). Sonication of the inactive homogenate from unfrozen vegetative cells is also effective to release and activate the vegetative form of the enzyme (Fig. 3). Since Claes (2) has used ultrasonication to disrupt cells, activity would be detectable in vegetative cells. That author has also reported as unpublished results (3) that gametes broken by the French press yield no activity unless the homogenate is subjected to sonication. In the present study, however, the above features apply to vegetative cells, but not to gametes. The reason for this discrepancy in the results remains obscure at present since we do not know her experimental details (3).

Since the vegetative form of the lytic enzyme is found mainly in the particulate fractions (15,000 to 30,000 g pellets) of cell homogenate (Table IV), the enzyme may be stored in the vesicles or granules in an inactive state. Freeze-thawing or sonication of cells or homogenates may result in the breakage of these vesicles or granules and release of the active enzyme into the soluble fraction. Another possibility is that the enzyme is inactivated in that it might be tightly attached to the cell structure such as the cell wall. An analogous situation is found in yeast (7, 8) and *Chlorella* (14), where the lytic enzymes show two forms during the cell cycle, the soluble form and the cell wall-associated form which is presumably the inactive one.

By contrast, the lytic enzyme of gametes may either be contained freely or associated loosely with the cell structures in that it can be solubilized and activated by French press dis-



**Figure 8.** Re-accumulation of lytic enzyme after wall regeneration. After treatment of vegetative cells with gamete wall-autolysin and washing out of the enzyme, protoplasts were incubated in culture medium from time zero shown on the abscissa. At the times indicated, aliquots were taken to determine the proportion of walled cells (*solid circles*) and lytic activity (*open circles*). The proportion of walled cells was quantified by the heating method (22). Lytic activity was assayed by use of homogenates from frozen cells.

ruption alone. In this study, we also analyzed the soluble acid phosphatase activity and find that this vacuolar enzyme (19) is released into the soluble fraction with similar ease by French press in vegetative cells and gametes (Table II). In addition, this enzyme comes out if only cells are frozen. On the other hand, homogenization by French press is required to solubilize the lytic enzyme even in gametes (Table II). We have shown previously that gamete wall-autolysin tends to form a large aggregate of 62-kD subunits in the culture medium or low salt buffer (23, 36). It is therefore possible that the acid phosphatase is released from gametic cells broken by simple freeze-thawing whereas the lytic enzyme remains in the cells due to the large size of the enzyme aggregate. Another possibility is that the lytic enzyme is associated with cellular components still in frozen gametes until the cells are homogenized drastically.

The present study also shows that homogenates or their soluble fractions of vegetative cells and gametes have only 20% of the lytic activity present in the medium of mating gametes (Tables I and II). This difference is due to the presence of some, if not all, inhibitor(s) of lytic activity in the soluble fraction. The inhibitor(s) is absorbed to DEAE-cellulose so that the total activity in the soluble fraction increases and reaches the level of the activity present in the mating medium. It is possible that the endogenous inhibitor acts on the lytic enzyme in cells to keep the activity below the critical level until its release by mixing opposite mating-type gametes.

### **The Storage Form of Lytic Enzyme Shifts via a Differentiation and Dedifferentiation Program**

Nitrogen starvation triggers in *C. reinhardtii* a gametogenic program (18) designed to acquire the flagellar agglutinability and fusing ability. According to this program, cells synthesize agglutinins in their cell bodies which are then transported to the flagellar surfaces for agglutination (21, 30) and construct the *mt<sup>+</sup>* or *mt<sup>-</sup>* mating structure for cell fusion (5). We predict from the present results that the gametogenic

program in *C. reinhardtii* also includes the event of the shift of the storage form of lytic enzyme. Upon nitrogen removal, the enzymatic behavior shifts from the vegetative state to the gametic state in parallel with the acquisition of mating ability (Fig. 5). Adding nitrogen back converts gamete to vegetative cell concurrently with the shift of the enzymatic behavior to the vegetative state (Fig. 6 and Table V). We therefore assume that the loss of mating ability and the return of the cell wall lytic enzyme to the vegetative state are both involved in a dedifferentiation program. Since cycloheximide appears to block the proceeding of the dedifferentiation program (Table V), the drug may prevent cells from responding to nitrogen addition.

Although vegetative cells and gametes of *C. reinhardtii* are morphologically very similar, one can now clearly distinguish between the two cell types by a simple determination of lytic activity by use of homogenates of unfrozen cells. This assay method will become a valuable tool to determine whether cells, which have been placed in a nitrogen starved condition, can truly differentiate into gametes, especially in cases of the non-agglutinating mutants or flagellar-deficient mutants.

### Lytic Enzyme Is Stored Outside the Plasmalemma

Finally, we suggest here that lytic enzyme is stored either in the periplasmic space or attached to the cell wall. Removal of the cell walls of vegetative cells or gametes by the exogenously added enzyme causes almost complete loss of the enzyme from the cells (Table VI). When these de-walled gametes are mixed together, they normally agglutinate and fuse, but release only very small amounts of the enzyme into the medium (Fig. 7). Similarly, in the wall-deficient mutants, *cw*, the amount of lytic activity in the homogenates of cells and in the medium after mating is practically negligible (Table VI and Fig. 7). These facts support that the lytic enzyme within the periplasmic space or in the cell walls and the gamete wall-autolysin released into the mating medium are identical.

When the de-walled vegetative cells are incubated after removing the exogenously added autolysin, the lytic enzyme is again accumulated in the cells after the reformation of the cell walls is almost complete (Fig. 8). Goodenough and Heuser (4) have analyzed the wall regeneration using the quick-freeze, deep-etch technique and shown that after lytic enzyme is washed away, cells quickly regrow radial fibers to constitute the "warp," and the elements of the central triplet are then assembled to form the "weft" within a period of a few hours. We therefore predict that the newly synthesized lytic enzyme would be organized outside the plasmalemma after the "weft" is formed.

In this organism, insoluble phosphatases and carbonic anhydrase are also located outside the plasmalemma (12, 13, 15, 19). As for the carbonic anhydrase, treatment of walled cells with trypsin causes a quantitative release of this enzyme into the medium, indicating that its locality is on the outer surface of the cell wall (38). We have used trypsin to examine its effect on the lytic enzyme in the cells. We find that treatment of gametes with trypsin does not cause any release of lytic activity from the cells, although this treatment has previously been shown to cause a complete release of agglutinins (30). Therefore, the lytic enzyme may not be located on the cell surface. Since the innermost layer of the central triplet

(W2 layer) has been proposed to be the target of lytic enzyme (4), it is plausible to assume that the enzyme is stored in the vicinity of the W2 layer. In this context, Millikin and Weiss (24) have analyzed the binding of Con A to the *Chlamydomonas* gametic cells and proposed that periplasmic Con A-binding sites represent lytic enzyme or a precursor of enzyme.

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