Regulated Secretion of a Serine Protease that Activates an Extracellular Matrix–degrading Metalloprotease during Fertilization in *Chlamydomonas*

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Abstract. During fertilization in the biflagellated alga, *Chlamydomonas reinhardtii*, gametes of opposite mating types adhere to each other via agglutinin molecules located on their flagellar surfaces, generating a sexual signal that induces several cellular responses including cell wall release. This cell contact–generated signal is mediated by cAMP and release of the wall, which is devoid of cellulose and contains several hydroxyproline-rich glycoproteins, is due to the activation of a metalloprotease, lysin. Although we originally assumed that lysin would be stored intracellularly in a compartment structurally separate from its substrate, recently we showed that lysin is stored in the periplasm as an inactive, higher relative molecular mass precursor, prolysin (Buchanan, M. J., S. H. Imam, W. A. Eskue, and W. J. Snell. 1989. *J. Cell Biol.* 108:199–207). Here we show that conversion of prolysin to lysin is due to a cellular, nonperiplasmic enzyme that has the properties of a serine protease. Release of this serine protease into the periplasm is induced by incubation of gametes in dibutyryl cAMP. This may be one of the few examples of regulated secretion of a protease in a eucaryotic microorganism and a novel example of regulated secretion in a plant system.

SPECIFIC cell–cell contacts play important signaling roles at several stages of development of multicellular organisms. This is especially true at fertilization, when contacts between gametes induce changes in intracellular levels of second messenger molecules (27, 28) leading to a series of responses in both gametes including changes in ion fluxes across the plasma membranes (27) and release and activation of stored hydrolytic enzymes (20, 27, 28). The released hydrolytic enzymes alter the extracellular matrix and cell surfaces of the interacting gametes in preparation for membrane fusion and zygote formation (2, 3, 20).

Many similar events also occur during fertilization in the biflagellated alga, *Chlamydomonas reinhardtii* (24). Flagellar adhesion between mt^+ and mt^- gametes initiates a sexual signal leading to formation of mating structures involved in cell fusion and activation of the cell wall degrading enzyme, lysin (7). *Chlamydomonas* is particularly useful for studies on cell contact–mediated signaling because it is a unicellular organism that is amenable to genetic analysis and mutants blocked at several stages of fertilization are already available (10). Fertilization is initiated when the flagella of mt^+ and mt^- gametes adhere to each other via adhesion molecules, agglutinins, that have been shown to be high molecular weight, extrinsic membrane proteins (1). This adhesive interaction is accompanied by a rapid increase in the concentration of intracellular cAMP (22). Recently, Pasquale and Goodenough (21) showed that many of the events that normally occur as a consequence of sexual signaling can be induced in cells of a single mating type by incubation of gametes in dibutyryl cAMP and a phosphodiesterase inhibitor.

Our laboratory has been interested in understanding the molecular details of activation of lysin, which is one of the earliest of these signaled events. It is known, from work by us and others (5, 12, 14), that the lysin released by mating gametes is a 60,000-M_r metalloprotease and recently we reported that it acts on a flagellar collar molecule as well as the highly cross-linked framework of the cellulose-deficient, glycoprotein-rich cell wall of *Chlamydomonas* (11). Other workers had presented evidence consistent with the idea that lysin was in the periplasm of gametes (18) and Matsuda et al. (15) reported that lysin could be isolated from homogenates of gametes. These workers found low levels of lysin activity in the original cell homogenate, but during purification there was about a fivefold increase in total activity.

Recently, using an antibody that we prepared against lysin, we compared the stored form of the enzyme to the form released by mating gametes (6). Immunoblots of intact gametes showed that the stored form of the enzyme was 62,000 M_r. On the other hand, the relative molecular mass of lysin released into the medium during mating was 60,000. The enzyme was not present in de-walled gametes, indicating that it is stored in the periplasm. In other experiments we found that the stored form of the enzyme, now called prolysin, did not have wall degrading activity. Since the released form of

lysin is active, the results suggested that the appearance of active lysin during mating might be a consequence of the appearance in the periplasm of an enzyme that converts prolysin to active lysin.

In the present report we show that gametes contain such a converting activity. The converting activity, designated *p*-lysinase, is distinct from lysin and remains in gametes whose walls and prolysin are removed by exogenous lysin. *p*-Lysinase has the properties of a serine protease and converts the inactive 62,000-M_r prolysin to active, 60,000-M_r lysin. *p*-Lysinase is released into the medium of de-walled gametes during the mating reaction as well as during incubation of mt^+ gametes in dibutyryl cAMP and a phosphodiesterase inhibitor and behaves on molecular sieve chromatography as a 300,000-M_r molecule. The results suggest that *p*-lysinase release is a regulated secretory event. This may be one of the few examples of regulated secretion in a plant system.

Materials and Methods

Cell Culture and Preparation of Gametes

Methods for cell culture were essentially as previously described (5). Gametogenesis was induced by transferring cells grown in acetate-supplemented medium to nitrogen-free medium diluted to 3/4 strength, and aerating in continuous light for 14–20 h. The resulting gametes were harvested by allowing them to accumulate at the bottom of the bottles by negative phototaxis/positive geotaxis (4) without aeration, and the supernatant was removed by siphoning. The gametes were further concentrated by centrifugation at 4,600 *g* for 3.5 min and then they were washed into Hepes-Ca^{2+} buffer (HC[1], 10 mM Hepes, 1 mM $CaCl_2$, pH 7.2). A hemocytometer was used to determine cell density.

Preparation of Lysin

Gametes were induced to release lysin into their medium as previously described (6). To do this, mt^+ and mt^- gametes were mixed together at a concentration of 3×10^8 cells/ml in HC buffer and incubated in bright fluorescent light for 30 min at 25°C. The cell suspensions were centrifuged at 4°C for 3.5 min at 4,600 *g*, the sedimented cells were discarded, and the supernatants were centrifuged at 4°C at 20,000 *g* for 30 min to remove cell debris and wall fragments. The lysin-containing supernatant was kept on ice or at 4°C until use. Samples not used immediately were divided into smaller portions and stored at −20°C.

Preparation of Prolysin

Prolysin was prepared as previously described (6). Briefly, gametes collected as above were frozen and thawed twice and the resulting high speed supernatant was used as a source of prolysin.

Electrophoresis and Immunoblotting

SDS-PAGE was carried out on 8% gels as previously described (5) and the gels were stained with silver (17). Antigens were transferred from polyacrylamide gels onto nitrocellulose paper at 300–400 mA for 2 h by the method of Towbin et al. (25). The anti-lysin first antibody was prepared in rabbits as previously described (6). Antigen-antibody complexes were visualized with peroxidase-conjugated goat anti-rabbit second antibody (Cappel Laboratories, Malvern, PA).

Assaying for p-Lysinase

p-Lysinase was assayed for by its ability to generate lysin from prolysin either by use of immunoblotting as described above or by a wall loss assay (5). To use the wall loss assay for *p*-lysinase, the sample containing *p*-lysinase was first incubated with prolysin at room temperature. Then, the lysin generated was assayed by use of the wall loss assay previously described (5). To do this 40 μl of tester vegetative cells (~2.5×10^7 cells/ml in HC) were incubated with 50 μl of sample for 15 min at room temperature. Then, 200 μl of ice-cold 0.075% Triton X-100, 5 mM EDTA in 10 mM Tris, pH 7.4, was added to solubilize the chlorophyl from the cells whose walls had been removed by lysin. After briefly vortexing and incubation for 10 min on ice, the samples were centrifuged and the OD_{435} of the supernatants was determined.

Results

Gametes Contain an Activity that Converts Prolysin to Lysin

Previously we showed that intact *Chlamydomonas* gametes contain only the inactive, higher relative molecular mass precursor of lysin, prolysin, which is stored in the periplasm (6). The observation that active lysin is generated during the mating reaction suggested that gametes store a converting activity in a separate cellular compartment. To determine if cells contained an activity that could convert endogenous prolysin to lysin, mating type plus (mt^+) gametes were disrupted by freezing and thawing twice followed by passage through a French pressure cell. The homogenate was incubated for 30 min at room temperature, centrifuged, and the supernatant was subjected to electrophoresis on SDS polyacrylamide gels followed by immunoblotting with an anti-lysin/prolysin antibody. A control sample of gametes was not subjected to French pressing, but was frozen and thawed twice and centrifuged. The control supernatant, which we have shown previously contains about one-half of the total cellular prolysin (6), was also prepared for immunoblotting. The results shown in Fig. 1 indicated that whereas the control sample contained prolysin (lane *1*), the homogenized sample contained only lysin (lane *2*), and no prolysin, indicating that the homogenate contained a converting activity. Initial characterization of this converting activity in cell homogenates showed that it was partially inhibited by 5 mM EDTA (Fig. 1, lane *3*), indicating that it required divalent cations.

Prolysin Converting Activity Is Released into the Medium during the Mating Reaction

We reasoned that since prolysin is converted to lysin during the mating reaction, an activity that carried out the conversion might be present in mating medium. When we tested this by incubating the medium of mating gametes with prolysin and assaying for its conversion to lysin using immunoblotting, activity (now designated *p*-lysinase) was detected (Fig. 1, lane *4*). To rule out the possibility that lysin itself had p-lysinase activity, the experiment was repeated with gametes whose walls and prolysin had been removed. To do this mt^+ and mt^- gametes were separately incubated with exogenous lysin as previously described. After washing, the cells were mixed to permit adhesion and sexual signaling and the supernatant was assayed for *p*-lysinase activity. As a control a sample of crude prolysin was incubated with buffer (Fig. 2, lane *1*) or with medium from nonmixed, de-walled gametes (Fig. 2, lane *3*). The results, shown in Fig. 2 (lane *2*), indicated that mating, de-walled gametes released *p*-lysinase, which converted the prolysin to lysin, whereas there was no conversion in either of the control samples.

1. *Abbreviation used in this paper*: HC, 10 mM Hepes, 1 mM Ca^{2+} Cl, pH 7.2.

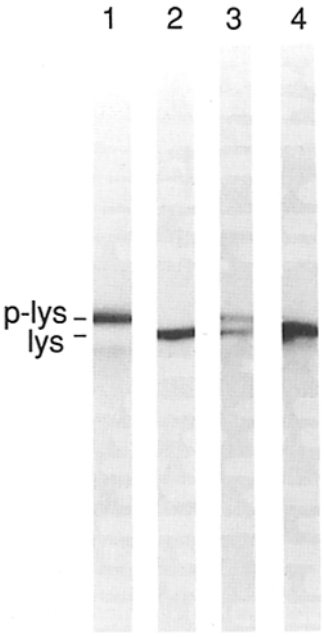

Figure 1. Gametes contain an activity that converts prolysin to lysin. Mt^+ gametes (1.6×10^8 cells/ml in HC) were either frozen and thawed twice (lane *1*) or frozen and thawed twice and then put through a French pressure cell at 20,000 psi (lanes *2* and *3*). After 30 min at room temperature the samples were centrifuged at 315,000 *g* for 10 min at 4°C, and 1 ml of the supernatant was lyophilized and resuspended in SDS-PAGE sample buffer for electrophoresis. EDTA (5 mM) was added to the sample in lane *3* before it was French pressed. The sample for lane *4* contained mating medium that was incubated for 30 min at room temperature with a sample of prolysin (6). Proteins were separated by SDS-PAGE in 8% gels, transferred to nitrocellulose, and immunoblotted with an anti-lysin/prolysin antibody. Prolysin (*p-lys*) is 62,000 M_r and lysin (*lys*) is 60,000 M_r.

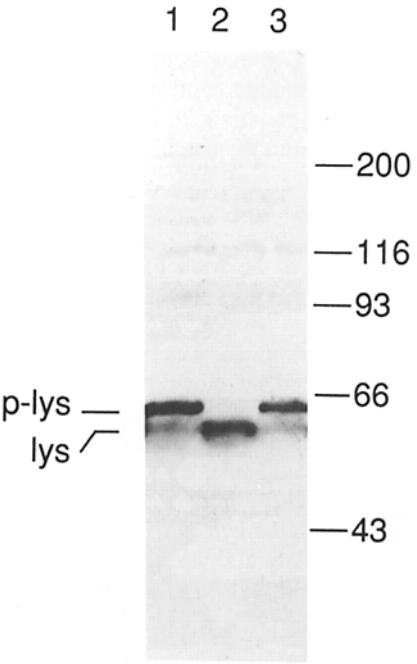

Figure 2. *p*-Lysinase released by mating gametes converts prolysin to 60,000 M_r lysin. Mt^+ and mt^- gametes were incubated with lysin to remove their walls and the de-walled gametes were mixed together for 5 min and centrifuged. This supernatant, along with control supernatants from nonmated mt^+ and mt^- gametes, was then tested for *p*-lysinase activity using immunoblotting. Lane *1* is an immunoblot of a sample of HC buffer incubated for 30 min at room temperature with prolysin. Lane *2* is the sample of mating medium incubated with prolysin and lane *3* is another control in which mt^- and mt^+ gametes were incubated separately for 5 min, centrifuged, and the two supernatants were combined and then incubated with a sample of prolysin. *p-lys*, prolysin; *lys*, lysin. Molecular weight markers are indicated on the right.

Confirmation that the 60,000-M_r lysin produced by *p*-lysinase was bona fide lysin was obtained by assaying the newly produced lysin for wall-releasing activity. To do this preparations containing prolysin or buffer were incubated with supernatants from nonmating or mating, de-walled gametes and then assayed in a wall loss assay (5). The results shown in Fig. 3 indicated that only the supernatant from mating gametes produced active lysin from prolysin. No lysin activity was detectable if prolysin was incubated with buffer or with medium from nonmating gametes. Nor was lysin activity present in the sample of mating medium that was mixed with buffer alone. Taken together these results provided evidence that mating gametes released an activity, distinct from lysin, that converted inactive prolysin to active lysin. The results also demonstrated that *p*-lysinase activity remained with the cells after wall removal. This indicated that *p*-lysinase is stored in a compartment distinct from prolysin, and is either bound to the cell surface or stored intracellularly.

p-Lysinase Has the Properties of a Serine Protease of ~300,000 Native Relative Molecular Mass

The availability of an enriched *p*-lysinase fraction from the medium of de-walled, mating gametes also made it possible to learn more about its properties. The result that *p*-lysinase caused a shift in relative molecular mass of prolysin suggested that *p*-lysinase might be a protease. For this reason we tested the effects of several protease inhibitors on *p*-lysinase using both the immunoblotting assay and the wall loss assay, and the results are shown in Table I. The effects of these inhibitors on lysin are also included for comparison. The results indicated that *p*-lysinase has the properties of a serine protease. It was inhibited by the serine protease inhibitors *N*-tosyl-$_L$-phenylalanine chloromethyl ketone, aprotinin, PMSF, and diisopropyl fluorophosphate. Whereas, it was not inhibited by the aspartic protease inhibitor, pepstatin, nor the cysteine protease inhibitor, *N*-ethylmaleimide, nor the metalloprotease inhibitor, phenanthroline. As Table I shows and has been reported by others (14), lysin has the properties of a metalloprotease in that it was inhibited by phenanthroline and none of the other specific inhibitors. EDTA and EGTA inhibited both enzymes, suggesting that divalent cations were necessary for activity.

Molecular sieve chromatography of partially purified *p*-lysinase was performed to obtain an estimate of native molecu-

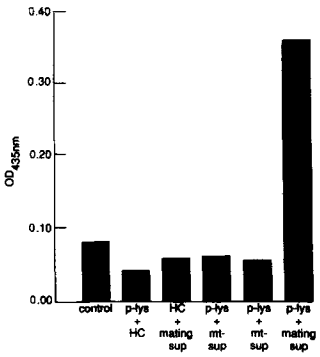

Figure 3. For the wall loss assay 40 μl of the supernatant from mating and nonmating gametes or HC buffer were incubated with 80 μl of prolysin in HC buffer prepared as above at room temperature as described in the text. An additional control sample contained mating supernatant but no prolysin. After 30 min at room temperature, 40 μl of tester mt^+ vegetative cells in HC were added to 50 μl of sample and incubated for 15 min at room temperature. The extent of wall loss was then determined as described in Materials and Methods.

Table I. Effects of Inhibitors on p-Lysinase Activity

Inhibitor	p-Lysinase	Lysin
	%	%
None	100	100
TPCK (*2 mM*)	10	95
Aprotinin (*0.1 mg/ml*)	4	95
DFP (*1 mM*)	12	83
NEM (*2 mM*)	+*	102
PMSF (*2 mM*)	±*	86
Pepstatin (*10 μg/ml*)	+*	78
Phenanthroline (*2 mM*)	+*	5
EDTA (*10 mM*)	−*	0
EGTA (*10 mM*)	−*	0

p-Lysinase was assayed either by immunoblotting as described in Fig. 1 legend or by its ability to generate active lysin, which was subsequently assayed by the wall loss assay described in Fig. 3 legend. +, *p*-lysinase was active in the presence of the inhibitor and all of the prolysin was converted to lysin. ±, partial activity. −, there was no conversion of prolysin to lysin in the presence of the inhibitor. Lysin activity was assayed by the wall loss assay. *TPCK*, *N*-tosyl-ₗ-phenylalanine chloromethyl ketone; *DFP*, diisopropyl fluorophosphate; *NEM*, *N*-ethylmaleimide.
* Experiments carried out by immunoblotting.

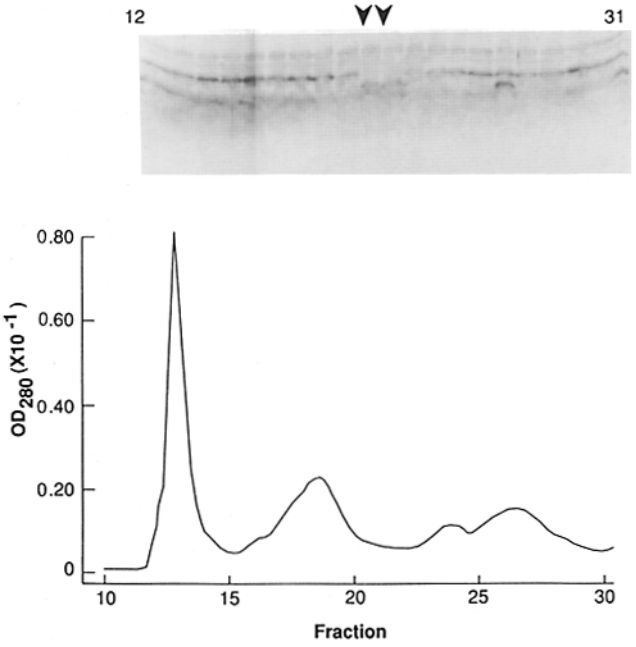

Figure 4. Native molecular weight of *p*-lysinase estimated from molecular sieve chromatography. Crude *p*-lysinase prepared from the medium of 280 ml of dewalled mating mt^+ and mt^- gametes (3×10^8 cells/ml in HC) was partially purified by ion exchange chromatography, concentrated, and separated on a column (model 4000SW; Beckman Instruments, Inc., Palo Alto, CA) using a high performance liquid chromatography system (Waters Instruments, Inc., Rochester, MN). The lower section shows the OD_{280} profile and the upper section shows an immunoblot of prolysin that had been incubated for 30 min with fractions 12–31 before SDS-PAGE. In the samples indicated by the arrowheads all of the prolysin had been converted to lysin. (The more weakly staining bands present in all of the samples are of unknown origin and significance.) By comparison with the elution of standard proteins (not shown), the native molecular weight of *p*-lysinase is ~300,000.

lar weight. Column fractions were assayed for prolyinase activity by incubating them with prolysin followed by immunoblotting. The results shown in Fig. 4 indicated that fractions 21 and 22 contained the majority of the prolyinase activity, consistent with a native molecular weight of ~300,000 compared to elution patterns of standard proteins of known molecular weight. Some lysin that contaminated this particular preparation of *p*-lysinase eluted in fractions 26 and 27, consistent with lysin's molecular weight of 60,000 (5, 12, 14).

Incubation of Gametes in Dibutyryl cAMP and Papaverine Induces Release of p-Lysinase

Finally, we wanted to determine if incubation of gametes under conditions that should raise their intracellular cAMP levels would induce release of *p*-lysinase. To do this de-walled gametes were incubated in dibutyryl cAMP and papaverine (a phosphodiesterase inhibitor), conditions that Pasquale and Goodenough (21) have reported induce sexual signaling, and the medium was assayed for *p*-lysinase activity. The results shown in Table II indicated that this treatment induced *p*-lysinase release from the nonmating gametes.

Discussion

Our results show that conversion of prolysin to the active wall-degrading enzyme, lysin, during fertilization in *Chlamydomonas* is due to the action of a cellular serine protease that we have designated *p*-lysinase. We first identified *p*-lysinase in cell homogenates by use of immunoblotting with an anti-lysin antibody. Intact gametes prepared for SDS-PAGE and immunoblotting under conditions where *p*-lysinase would not be in contact with prolysin were shown to contain only the 62,000-M_r prolysin. Whereas, if homogenates of gametes were incubated for 30 min at room temperature before SDS-PAGE and immunoblotting, all of the prolysin was converted to the 60,000-M_r lysin.

By use of the immunoblotting assay we also showed that *p*-lysinase was released into the medium during the mating reaction. The result that de-walled gametes also released *p*-lysinase indicated that, unlike prolysin, this enzyme was not stored in the periplasm. Moreover, *p*-lysinase released into the medium by mating, de-walled gametes was free of lysin, making it possible to use a wall loss assay to show that the 60,000-M_r form of lysin generated by *p*-lysinase was bona fide lysin and had wall-degrading activity.

Table II. Effect of Dibutyryl cAMP and Papaverine on p-Lysinase Release from mt^+ Gametes

Sample	*p*-Lysinase (OD_{435})
Pipes	0.009
Pipes and DMSO	0.016
Dibutyryl cAMP (*10 mM*), papaverine (*0.1 mM*)	0.074

De-walled mt^+ gametes (1×10^8 cells/ml in 10 mM Pipes, pH 7.8) were incubated for 35 min at room temperature in the indicated concentrations of dibutyryl cAMP and papaverine. Control samples were incubated in Pipes alone or Pipes with 2% DMSO, the solvent for the papaverine. At the end of the incubation the samples were centrifuged, the supernatants concentrated with Centricon filters (Amicon) and assayed for *p*-lysinase in the wall loss assay.

Our observations on the conversion of prolysin to lysin are consistent with results from Matsuda et al. (15) who reported that they were unable to obtain lysin from nonmating gametes unless the cells were first homogenized. Even after homogenization this group obtained only 20% of the lysin activity that was released into the medium by the same number of mating gametes. As we showed previously prolysin is released from gametes if they are frozen and thawed twice (6). But under these conditions none of the prolysin is converted to lysin. On the other hand, as described above, homogenization and incubation at room temperature for 30 min was sufficient to convert all of the prolysin to lysin. The fact that Matsuda et al. carried out their homogenization at 4°C might explain why they had incomplete conversion of prolysin to lysin. Furthermore, these workers reported that there was a fivefold increase in lysin activity detected after partial purification on ion exchange resin. They interpreted the result to mean that ion exchange had removed an inhibitor. If this is true, prolysin might have been the inhibitor, which was separated from lysin during ion exchange chromatography. In preliminary experiments (not shown) we have found that lysin activity is inhibited by large amounts of prolysin. This is probably due to the ability of prolysin to bind to lysin's substrate without degrading it. An alternative explanation for the increased activity of lysin after purification by ion exchange that Matsuda et al. reported could also be that the time it took to carry out the purification step permitted more prolysin to be converted to lysin.

Further characterization of *p*-lysinase indicated that it had the properties of a serine protease. It was inhibited by several serine protease inhibitors and not by other types of protease inhibitors including phenanthroline, a metalloprotease inhibitor that completely inhibits lysin (14, 16).

Our results on the conversion of this extracellular *Chlamydomonas* prometalloprotease to the active enzyme is similar in many respects to observations on the major metalloproteases in higher organisms, the collagenases. As with lysin, most mammalian collagenases are also released into pericellular and extracellular spaces as inactive, higher molecular weight precursors (9). The in vivo mechanism(s) of activation of the precursors has not yet been clearly identified (29). In vitro, organic mercurials can activate by their ability to bind to a free sulfhydryl, permitting a conformational change that ultimately leads to autoconversion of procollagenase (9). Several serine proteases including kallikrein (19) and plasmin (8) have converting activity in vitro and have been suggested as possible in vivo activators. And recently Unemori and Werb showed, using rabbit synovial fibroblasts that were induced to produce active collagenase, that addition of serine, thio-, and metalloprotease inhibitors prevented conversion to varying degrees, suggesting that there may be a cascade of proteolytic events leading to activation of collagenase (26). It will be interesting to compare the amino acid sequence of lysin, once it is available, to the known sequences of procollagenases to determine if the functional similarities extend to the molecular level.

Initial determination of the native molecular weight of lysin suggested that it was about 300,000 M_r and also showed that it was clearly separable from lysin. We are currently developing methods for purification of *p*-lysinase, which will permit us to learn more about its physical properties.

Finally, an especially interesting aspect of this work is that release of *p*-lysinase can be induced by incubation of gametes in dibutyryl cAMP and papaverine. Although we recognize that there may be other possibilities for release (13), we feel that the simplest explanation for these results is that *p*-lysinase is stored in an intracellular vesicle whose exocytosis is induced by increased levels of cAMP. To our knowledge this is a novel example of regulated secretion in a plant system.

These results indicate that *Chlamydomonas* gametes contain a cellular serine protease whose release is induced by the sexual signaling that occurs during the mating reaction. The requirement for cell contact can be abrogated by incubation of the cells in dibutyryl cAMP and papaverine. This newly described example of regulated secretion in *Chlamydomonas* should make it possible to learn more about the molecular mechanisms of signal transduction and regulated secretion mediated by specific cell contact. In addition, since *Chlamydomonas* is especially amenable to genetic manipulation, it should also be possible to study the molecular signals responsible for sorting prolysin and *p*-lysinase into their distinct cellular compartments along the secretory pathway.

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