

Activation of the Cell Wall Degrading Protease, Lysin, during Sexual Signalling in *Chlamydomonas*: The Enzyme Is Stored as an Inactive, Higher Relative Molecular Mass Precursor in the Periplasm

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Abstract. During the mating reaction in *Chlamydomonas reinhardtii* mating type plus and mating type minus gametes adhere to each other via adhesion molecules on their flagellar surfaces. This adhesive interaction induces a sexual signal leading to release of a cell wall degrading enzyme, lysin, that causes wall release and degradation. In this article, we describe the preparation of a polyclonal antibody against the 60,000- M_r lysin polypeptide excised from SDS-PAGE gels. After absorption of the IgG with cell walls to remove antibodies against a carbohydrate epitope common to several *Chlamydomonas* glycoproteins, the immune IgG reacted with the 60,000- M_r polypeptide, and a 47,000- M_r species that we show here was immunologically cross-reactive with the 60,000- M_r molecule. By use of several fractionation methods including ion exchange and molecular sieve chromatography, sucrose gradient centrifugation, and affinity chromatography, we showed that the 60,000- M_r antigen copurified with lysin activity, thereby demonstrating that the antibody

was indeed directed against the enzyme.

Immunoblot experiments on suspensions of nonmating and mating gametes showed that the 60,000- M_r antigen was missing in the nonmating gametes. Instead, they contained a 62,000- M_r antigen that was not present in suspensions of mating gametes that had undergone sexual signalling. Furthermore, nonmating gametes whose walls were removed with exogenously added lysin did not contain either form of the antigen. We also found that the 62,000- M_r form of the antigen, which could be released from gametes by freeze-thawing, did not have wall degrading activity. These results indicate that lysin in gametes is stored in the periplasm as a higher relative molecular mass, inactive precursor and also that sexual signalling induces conversion of this molecule to a lower relative molecular mass, active enzyme. This may be a novel example of processing of an extracellular protease induced by cell contact.

IN the biflagellated alga, *Chlamydomonas reinhardtii*, fertilization is initiated when mating type plus (mt+)¹ and mating type minus (mt-) gametes adhere to each other via their flagella. This specific cell-cell recognition event induces a sexual signal that leads to several subsequent events including release of an enzyme, lysin, that causes wall shedding and degradation (10, 36, 40). Although the flagellar surface molecules responsible for the initial adhesive interaction have been identified (1), until recently there has been little information available about the mechanism of sexual

signalling. Our laboratory has shown that the local anesthetic, lidocaine, blocks signalling without interfering with flagellar adhesion (37), and others have shown that trifluoperazine has similar effects (8). Bloodgood and Levine (3) have shown that the mating reaction is accompanied by a rise in the rate of calcium release from cells and Kaska et al. (17) have shown mating-dependent changes in intracellular Ca^{2+} . The van den Ende laboratory (30) reported that there is a transient rise in the concentration of intracellular cyclic AMP during mating. And more recently Pasquale and Goodenough (29) made the exciting observation that cyclic AMP in combination with phosphodiesterase inhibitors was able to induce sexual signalling in gametes of a single mating type of *Chlamydomonas reinhardtii*.

To learn more about the molecular details of sexual signalling we have decided to characterize one of the earliest signalled events, release of the cell wall degrading enzyme,

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1. *Abbreviations used in this paper:* HC, HEPES- Ca^{2+} (10 mM HEPES, 1 mM $CaCl_2$, pH 7.2); mt-, mating type minus; mt+, mating type plus; r-lysin, released lysin; s-lysin, stored lysin.

lysin. This enzyme appears in the medium within 1–2 min after mixing gametes of opposite mating types at about the same time that the interacting gametes shed their walls into the medium. Presumably, when lysin is first activated or released, it acts on the intact wall to cause wall shedding. After the wall has been shed, the activated lysin appears in the medium (6, 18, 31, 34).

Jaenicke et al. (15) and Schlosser (31) have also described a separate lysin, sporangial lysin, that is responsible for degrading the sporangial cell wall and releasing the daughter cells arising during division. In contrast to the lysin released during mating, the sporangial lysin works only on the sporangial cell wall and is not released during the mating reaction. The sporangial lysin has a M_r of $\sim 40,000$ (15).

Recently, more information has become available about the lysin released during mating and its mode of action on the cellulose-deficient cell wall of *Chlamydomonas*. Millikin and Weiss (22) and Matsuda et al. (20) have presented evidence that the enzyme is stored outside of the plasma membrane, possibly in the periplasm. These workers showed that cells without walls do not contain lysin. Other studies on the enzyme indicate that the enzyme is a metalloendoprotease that acts upon several highly insoluble molecules in the wall (13, 14, 19, 23), one of which is in the flagellar collar (13, 35). Matsuda et al. (18) reported that lysin was a molecule of $\sim 60,000 M_r$, and our laboratory (4) and Jaenicke et al. (15) obtained similar results using independent purification and assay methods.

Subsequent analysis by our laboratory (4) and Jaenicke et al. (15) of the nondenatured enzyme demonstrated that it was a monomer. Having developed these new methods for purification of the enzyme, it became possible to learn more about the molecular basis of its release during the mating reaction. In the present report we describe the preparation and characterization of an antibody against the 60,000- M_r enzyme released into the medium by mating gametes. By use of the antibody we made the surprising observation that the enzyme is stored as an inactive, higher relative molecular mass precursor in the periplasm. This may be one of the few examples of a zymogen in a eucaryotic microorganism (26).

Materials and Methods

Cell Culture and Preparation of Lysin

Methods for cell culture were essentially as previously described (33). 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 (2, 14) 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; 10 mM HEPES, 1 mM CaCl_2 , pH 7.2). A hemocytometer was used to determine cell density.

To induce gametes to release lysin into their medium, 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. After 30 min the percentage of cells forming zygotes was determined as previously described (37). Only preparations showing >70% fusion were used. 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 immedi-

ately were divided into smaller portions and stored at -20°C. Lysin activity was measured as previously described (4).

Antibody Production and Preparation

Partially purified lysin was concentrated in dialysis bags with Aquacide II (Calbiochem-Behring Corp., La Jolla, CA) and subjected to electrophoresis on preparative 4–16% polyacrylamide gels containing a three to eight molar gradient of urea (16). After staining with Coomassie Brilliant Blue, the band migrating at 60,000- M_r was excised, equilibrated with PBS (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2), and homogenized with a glass-teflon homogenizer. The homogenate was emulsified by sonication in an equal volume of Freund's complete adjuvant and injected intradermally, intramuscularly, and subcutaneously into six to nine pound New Zealand white rabbits. Antisera were obtained from clotted blood by centrifugation at 3,000 g for 10 min.

To prepare the IgG fraction, antiserum was brought to 45% saturation with ammonium sulfate at 25°C, stirred for 20 min, and centrifuged 10,000 g for 30 min at 4°C. The precipitate was washed once with 45% ammonium sulfate and dialyzed against phosphate buffer (17.5 mM, pH 6.3). The IgG fraction was further purified either by ion exchange chromatography or protein A affinity chromatography.

Preparation of Cell Walls for Antibody Absorption

Cell walls obtained from mechanically disrupted *Chlamydomonas* gametes as previously described (14) were used to absorb unwanted antibodies from the immune IgG. Sedimented walls were stored in 0.1% sodium azide in HC at 4°C. The preparation of immune IgG was absorbed by incubating 4 mg antibody protein with 1 mg wall protein in 1 ml of PBS. The walls and bound antibodies were removed by centrifugation.

Preparation of a Substrate Affinity Column

A substrate affinity column was prepared by derivatizing Biogel A-15M (Bio-Rad Laboratories, Richmond, CA) with cell wall frameworks, which we have shown contain the substrate for lysin (13, 14), prepared from mechanically isolated cell walls. To do this, walls obtained from $\sim 2 \times 10^{11}$ cells were extracted by incubating them in 20 ml of SDS-PAGE sample buffer for 1 h at room temperature. This treatment removed all of the peripheral wall proteins but left the framework of the wall (13). The suspension was then centrifuged at 20,000 g for 20 min at 4°C and the sample was reextracted and harvested again. The sedimented frameworks were resuspended in HC and washed three times by centrifugation. Any SDS associated with the frameworks was removed by washing twice with 100% ethanol. Frameworks were washed back into 7 ml of HC and sonicated for 3.5 min on ice with the maximum setting of a Sonifier cell disrupter (model W185; Heat Systems-Ultrasonics, Inc., Farmingdale, NY). The sonicated suspension was coupled to 50 ml of Bio-gel A-15 agarose beads by the method of Nilsson and Mosbach (25) for 10 h at 4°C with agitation. Unreacted groups were blocked by washing the beads into 0.2 M Tris, pH 8.0, followed by incubation in the same buffer at 4°C for 10 h. The gel was washed twice with HC and stored at 4°C with 0.02% sodium azide until use.

Electrophoresis and Immunoblotting

SDS-PAGE was carried out according to the method of Jarvik and Rosenbaum (16). The running portion of the gels consisted of either 4–16% gradients of acrylamide or straight 8 or 10% acrylamide. Gels were stained with silver (21) or Coomassie Brilliant Blue (9). M_r were obtained by regression analysis from plots of \log_{10} percent acrylamide concentration vs. \log_{10} molecular mass for marker proteins (11). Antigens were transferred from SDS polyacrylamide gels onto nitrocellulose paper at 300–400 mA for 2 h by the method of Towbin et al. (39). Antigen-antibody complexes were visualized with peroxidase-conjugated goat anti-rabbit second antibody (Cappel Laboratories, Malverne, PA).

In some experiments rabbit antibodies were eluted from pieces of nitrocellulose blots by the method of Olmstead (27), neutralized with sodium hydroxide, diluted with PBS, and concentrated with millipore CX-30 filters. For staining blot strips, concentrates were rediluted with blocking buffer containing 10 mM Tris, pH 7.2, 150 mM NaCl, 0.2% NP-40, and 5% BSA (5).

Results

Preparation of Anti-Lysin Antibodies

Recently, using entirely different purification methods, we and others (4, 15, 20) have found that lysin is a polypeptide of $\sim 60,000 M_r$. Fig. 1, a preparative SDS-PAGE gel of lysin purified by ion exchange chromatography (4), shows that the $60,000-M_r$ polypeptide was the primary constituent of lysin purified by our method. This band was excised from the gel and used as immunogen in a rabbit as described in Materials and Methods.

Initial immunoblots were done with a preparation of crude lysin to determine the number of polypeptides that would react with the IgG fraction of the antiserum. Fig. 2, lane A shows a silver-stained gel of crude lysin. Lysin, at $60,000 M_r$, was a minor constituent of this preparation. The major constituents of the sample were cell wall proteins, which are also released into the medium during the mating reaction. Immunoblot analysis of the crude lysin showed that there was no staining with an irrelevant IgG (Fig. 2, lane B), whereas the immune IgG gave a complex staining pattern with many polypeptides reacting with the antibodies (lane C). The staining of multiple bands with the immune IgG was not an unexpected result, since we and others (1, 32, 41) have found that a carbohydrate determinant common to many *Chlamydomonas reinhardtii* glycoproteins is highly immunogenic in several species. To remove the antibodies against this common epitope the IgG preparation was absorbed (see Materials and Methods) with isolated cell walls, which are enriched with glycoproteins containing the epitope (32, 41). Fig. 2, lane D shows that the absorption yielded a highly specific antibody that stained polypeptides of $60,000$ and $47,000 M_r$ in a sample of crude lysin.

Previously, we had found that some lysin preparations contained a $47,000-M_r$ species (4) that varied in amount from experiment to experiment. Because we suspected that the $47,000-M_r$ polypeptide was a proteolytic fragment of the $60,000-M_r$ species, we wanted to determine if antibodies raised against the two polypeptides were cross-reactive. To

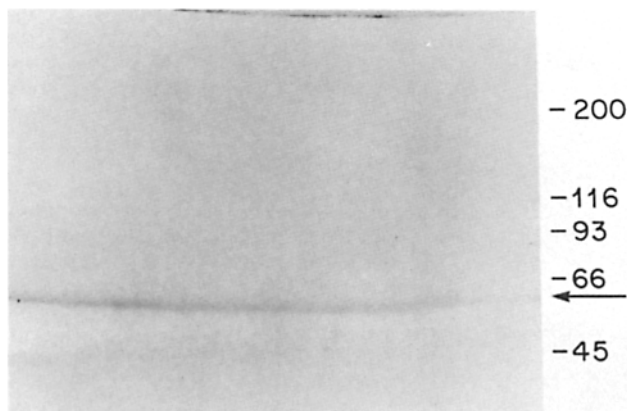


Figure 1. Preparative SDS-PAGE of carboxymethyl cellulose-purified lysin stained with Coomassie Brilliant Blue. The arrow indicates the $60,000-M_r$ lysin polypeptide that was excised from this and similar gels and used as immunogen in the preparation of the anti-lysin antibody. Relative molecular mass markers are indicated on the right.

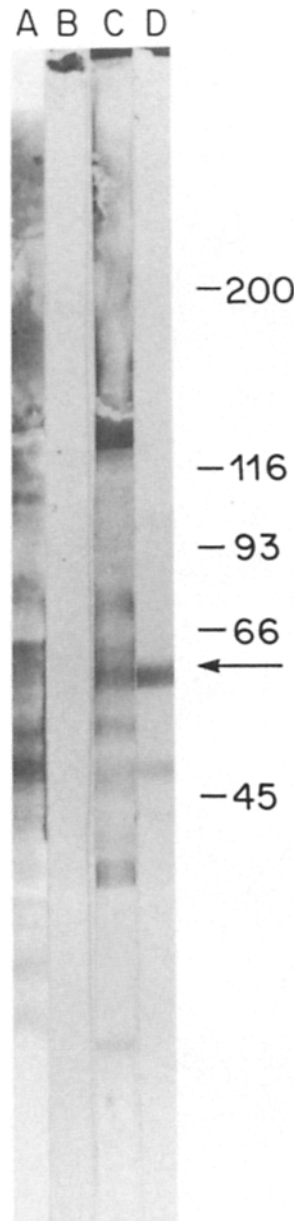


Figure 2. Absorption of the anti-lysin antibody with cell walls. Crude lysin was subjected to electrophoresis on a preparative SDS-PAGE gel and the polypeptides were transferred onto nitrocellulose paper. (Lane A) An untransferred portion of the SDS-PAGE gel was stained with silver to show the polypeptide composition of crude lysin. Most of the polypeptides in this heavily loaded gel were cell wall glycoproteins shed into the medium during the mating reaction. (Lanes B-D) For immunoblot analysis the nitrocellulose paper was cut into strips and incubated with (lane B) irrelevant IgG, (lane C) immune IgG from the rabbit immunized with the $60,000-M_r$ polypeptide, and (lane D) immune IgG absorbed with cell walls (see Materials and Methods), and then stained with a peroxidase-conjugated second antibody. Relative molecular mass markers are indicated on the right; the arrow indicates the $60,000-M_r$ lysin polypeptide.

do this we used the method of Olmstead (27) to examine if antibodies that bound to the $60,000-M_r$ would also bind to the $47,000-M_r$ molecule and vice versa. Two pools of antibodies were obtained by incubating blot strips of crude lysin with the absorbed, immune antibody and then separately excising the $60,000-$ and $47,000-M_r$ regions from the nitrocellulose strips as described in Materials and Methods. The antibodies eluted from each of the excised pieces of nitrocellulose were then incubated with fresh blot strips of crude lysin. As shown in Fig. 3 antibodies that initially bound to either the $60,000-$ or the $47,000-M_r$ polypeptides were each capable of rebinding to both polypeptides. Fig. 3, lane A shows a blot strip of lysin stained with an irrelevant antibody and lane B shows the staining pattern with the absorbed IgG. Lane C is a lysin blot stained with antibodies eluted from the $60,000-M_r$ band and lane D is an identical blot stained with antibodies eluted from the $47,000-M_r$ band. These results

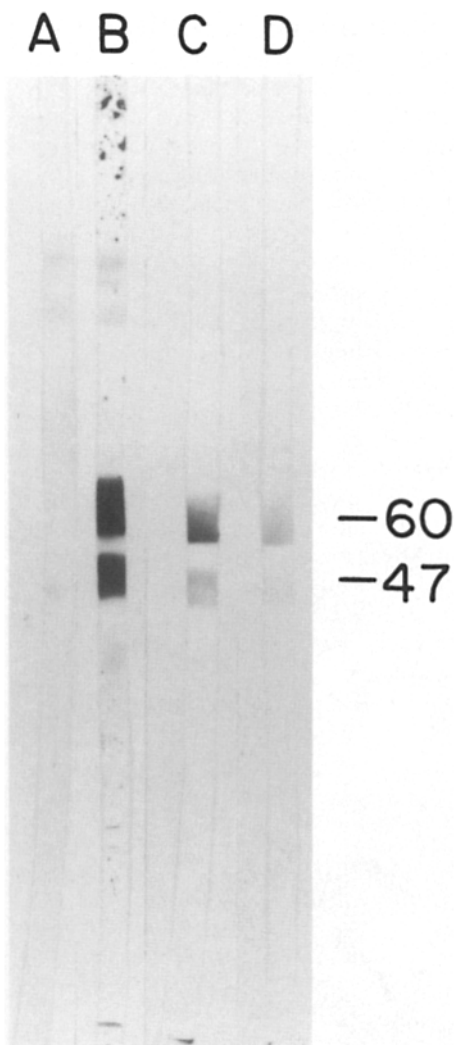


Figure 3. Immunocross-reactivity of the 60,000- and 47,000- M_r polypeptides. The absorbed, anti-lysin IgG was incubated with nitrocellulose blots of lysin and a portion of the blot was stained as described in Materials and Methods to localize the 60,000- and 47,000- M_r bands. Lane *A* shows a strip stained with an irrelevant antibody and lane *B* shows the staining with the anti-lysin antibody. The 60,000- and 47,000- M_r regions of the remainder of the blot, containing the bound antibody, were separately excised and the bound antibodies were eluted. The antibodies eluted from each band were then used to immunostain fresh nitrocellulose strips of lysin. Lane *C* shows the staining pattern with the antibodies eluted from the 60,000- M_r band and lane *D* shows the staining pattern with the anti-47,000- M_r antibodies. Antibodies eluted from each band reacted with both bands on the fresh strips.

suggested that the 47,000- M_r species was a proteolytic fragment of the 60,000- M_r molecule. In the experiments shown below, the 47,000- M_r fragment rarely appeared, probably because most samples were boiled in SDS-PAGE buffer immediately after preparation.

Lysin Activity Copurifies with the 60,000- M_r Antigen

The specificity of the IgG for lysin was evaluated by comparing the behavior of lysin activity with the behavior of the 60,000- M_r antigen both in cell suspensions and in several fractionation methods. For example, if the antigen were in-

deed lysin, it should be released into medium during the mating reaction between mt+ and mt- gametes. Fig. 4 shows that the antigen was released into the medium during mating (lane *B*) and was not present in the medium of nonmating gametes (lane *A*).

Several biochemical methods were also used to confirm the identity of lysin activity and the 60,000- M_r antigen. Fig. 5 demonstrates that the antigen and lysin activity coeluted in fractions obtained from a carboxymethyl cellulose ion exchange column. Fig. 6 shows the sedimentation pattern of lysin and the 60,000- M_r antigen in 5-20% sucrose gradients. The antigen and activity had identical sedimentation values. It should be noted that in this gel the 60,000- M_r antigen appeared as a doublet (see below). Fractionation with an HPLC molecular sieve column also showed that the antigen and lysin activity copurified (not shown).

To further evaluate the specificity of the antibody for lysin, a method was used that took advantage of the activity of the enzyme. Our laboratory has previously shown that a highly insoluble portion of the *Chlamydomonas* cell wall, called the framework, contains substrates for lysin (13, 14). Although this portion of the wall represents only 10% of the protein of the wall, it is the only part of the wall that is acted upon by lysin. The outer or peripheral portion of the wall contains several soluble glycoproteins, but these molecules do not appear to be lysin substrates (13, 19, 34). With this in mind, frameworks prepared from walls isolated from mechanically disrupted gametes were coupled to Biogel A-15M, as described in Materials and Methods, and used as an affinity matrix.

Lysin prepared by ion exchange chromatography was loaded onto the column, followed by washing with HC. The bound material was then eluted with NaCl, which has been shown by Matsuda (18) to inhibit lysin activity. The resulting fractions were dialyzed, tested for activity in the lysin assay, and immunoblotted. The results shown in Fig. 7 indicated that both lysin and the 60,000- M_r eluted as a sharp, coincident peak. The simplest interpretation of these results that lysin activity and the 60,000- M_r antigen could not be separated in any of the several independent methods described above was that the 60,000- M_r antigen was indeed lysin.

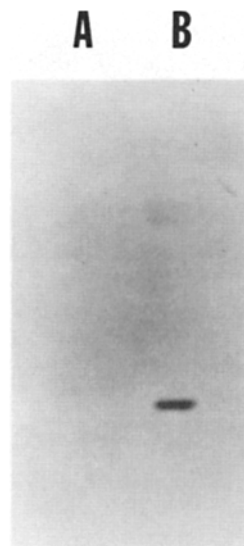


Figure 4. Release of the 60,000- M_r antigen during mating. Mt+ and mt- gametes were mixed together and the supernatant was collected and immunoblotted with the anti-lysin antibody (lane *B*). Lane *A* shows an immunoblot of a mixture of supernatants of mt+ and mt- gametes before mating. The arrowhead indicates the 60,000- M_r antigen.

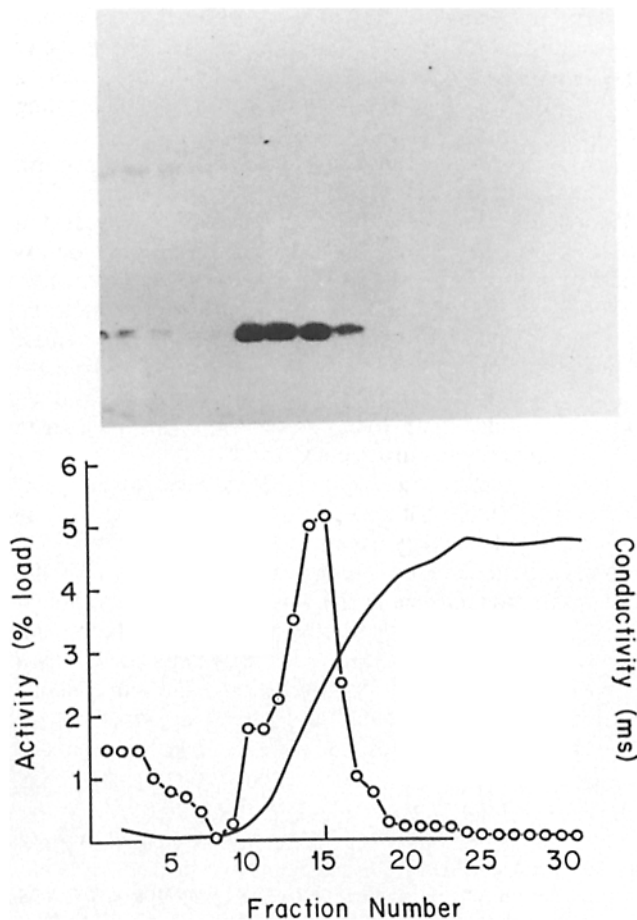


Figure 5. Cofractionation of lysin activity with the 60,000- M_r antigen on ion exchange chromatography. Crude lysin (100 ml; see Materials and Methods) was loaded onto a 50-ml carboxymethyl cellulose column equilibrated with HC, washed through with 10 ml of HC, and eluted with 100 ml of HC containing 0.2 M NaCl. Fractions (3.45 ml) were collected, and, after dialysis against HC, samples were assayed for lysin activity (bottom, \circ ; the solid line shows the conductivity of the fractions). Lysin activities are plotted as a percentage of loaded activity per fraction. (Top) Samples from each fraction were immunoblotted onto nitrocellulose and stained with anti-lysin antibody. The 60,000- M_r antigen copurified with activity.

Even though the anti-lysin antibody had been made against an SDS-DTT denatured form of the antigen, it was possible that the antibody would react with the native form. Several approaches were used to test this with the following results: The anti-lysin antibody did not inhibit lysin activity; affinity columns prepared with the antibody did not bind the native antigen; and dot blot experiments also showed that only the denatured form of the antigen was recognized by the antibody (data not shown).

Stored Lysin Has a Higher M_r Than Lysin Released during Mating

Having characterized the antibody it became possible to learn more about the stored form of the enzyme. To do this, dense suspensions of mt+ and mt- gametes were prepared and mixed together either before or after being boiled in SDS-PAGE sample buffer. The sample boiled before mixing the gametes would contain stored lysin, whereas the sample

boiled after mixing would contain the released form of the enzyme. The immunoblot results are shown in Fig. 8. Lane A, containing the sample boiled before mating, showed a band of slightly higher relative molecular mass than the sample that was boiled after mating (lane B). These results indicated that stored lysin (s-lysin), the form found in unmated gametes, was of slightly higher relative molecular mass than released lysin (r-lysin), which appeared in the medium during mating.

A separate suspension of mating gametes was centrifuged to separate the cell bodies from the mating supernatant, which contained released lysin. Analysis of these samples by immunoblotting showed that the r-lysin was in the medium (Fig. 8, lane C), and that the mated cells no longer contained detectable s-lysin (lane D). These results, which were consistent with previous results from our laboratory (34) that showed that mating gametes released all of their lysin activity within the first few minutes of mating, also demonstrated that release of active lysin during mating was accompanied by a reduction in its relative molecular mass.

Although the 4-16% gels used in these experiments resolved s-lysin from r-lysin, we occasionally noticed that r-lysin appeared as a doublet (see Fig. 6). When samples were run on straight 10% gels, however, their doublet nature

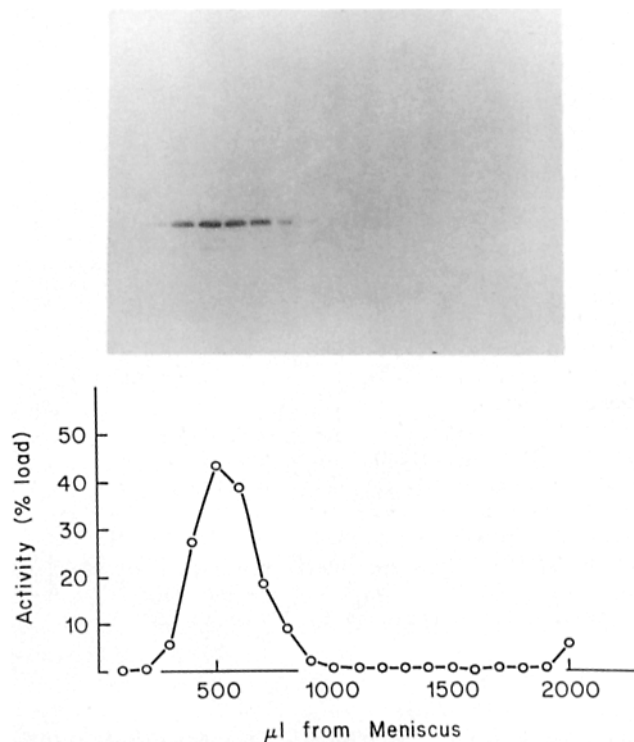


Figure 6. Copurification of lysin activity and the 60,000- M_r antigen on sucrose gradients. Lysin (20 ml) purified by carboxymethyl cellulose chromatography, as described in Materials and Methods, was concentrated to 0.2 ml and loaded onto a 2.0-ml 5-20% linear sucrose gradient. The gradient was centrifuged 5 h at 75,000 g and fractionated into 0.1-ml fractions that were dialysed, assayed for activity, and immunoblotted. (Top) Immunoblot showing the 60,000- M_r antigen. The fraction numbers are indicated in the bottom section. (Bottom) Lysin activity (as a percentage of loaded activity) in each of the fractions.

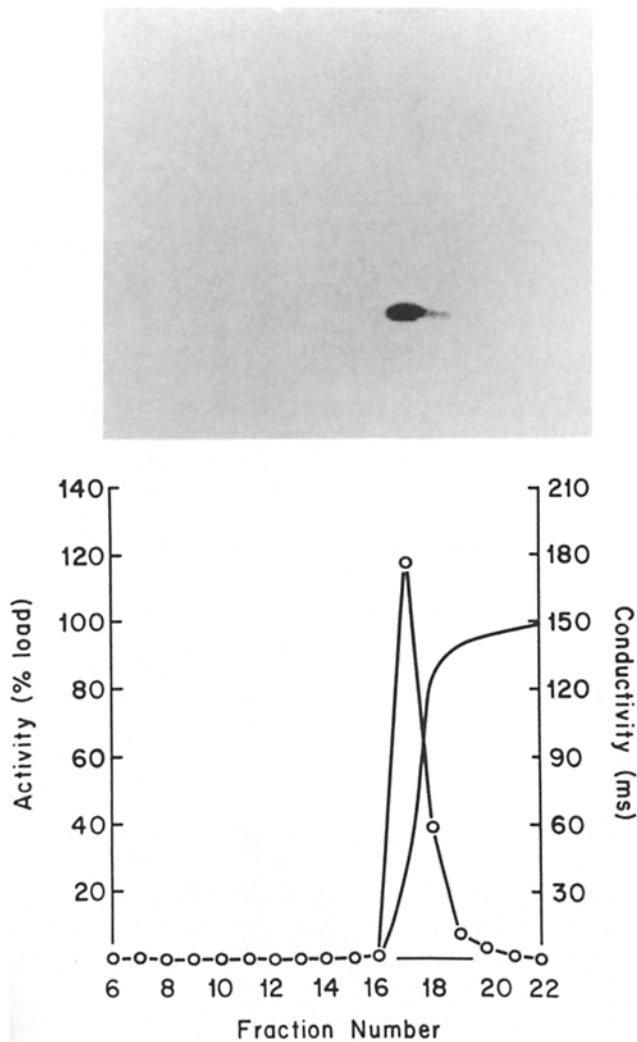


Figure 7. Copurification of lysin activity and the 60,000- M_r antigen on a substrate affinity column. Carboxymethyl cellulose-purified lysin was loaded onto an affinity column prepared with the framework fraction of *Chlamydomonas* cell walls as described in Materials and Methods. After washing the column with HC, it was eluted with HC containing 0.2 M NaCl and fractions were analysed for activity (bottom) and the 60,000- M_r antigen (top). The solid line indicates the conductivity of the fractions. Almost all of the activity and the 60,000- M_r antigen coeluted in fraction 17.

became clearer. Fig. 9 presents the results of an immunoblot experiment in which samples containing either s-lysin or r-lysin (prepared as described for Fig. 8) were loaded in separate lanes (Fig. 9, lanes A and C, respectively) and as a mixture (Fig. 9, lane B). The results indicated that both s-lysin (lane A) and r-lysin (lane C) were doublets. (The closed circles indicate the members of the s-lysin doublet, and the asterisks indicate those of the r-lysin doublet.) Lane B shows the mixture of the two samples and indicates that all four polypeptides were distinguishable.

Since the samples used as a source of s-lysin and r-lysin in this experiment contained a mixture of mt+ and mt- gametes, we wanted to learn if mt+ and mt- gametes each had a unique form of s-lysin. The other possibility was that cells of each mating type contained s-lysin doublets. To resolve this issue, immunoblots were done on unmated gametes of

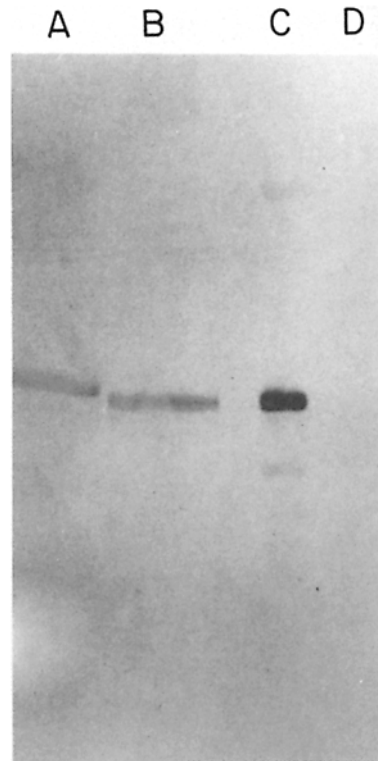


Figure 8. The stored form of lysin is of higher relative molecular mass than the released form. Suspensions of mt+ and mt- gametes (1 ml each; 8×10^8 cells) were mixed together either before or after being boiled in SDS-PAGE sample buffer in preparation for electrophoresis on 4-16% gradient gels. The samples mixed together for 30 min before being boiled would have the released form of lysin (r-lysin). The samples mixed together after being boiled would have the stored form of lysin (s-lysin). Lane A shows an immunoblot with the anti-lysin antibody of the stored lysin. Lane B shows the sample with the released lysin. The stored lysin was of slightly higher relative molecular mass than the released lysin. An identical sample of mt+ and mt- gametes that had been mating for 30 min was centrifuged at 10,000 g for 60 s, and the sedimented cells and the supernatant were immunoblotted with the anti-lysin antibody to determine the location of the antigen after mating. Lane C shows the mating supernatant and lane D shows the sedimented mated cells. The cells had released all of their lysin in to the medium as r-lysin and no longer contained s-lysin.

each mating type. The results, shown in Fig. 9 (lanes D and E), indicated that each gamete contained a single form of lysin and that the s-lysin in mt+ gametes (lane D) was of slightly higher relative molecular mass than that present in mt- gametes (lane E).

S-Lysin Is Stored in the Periplasm and Is Inactive

To learn more about the cellular site for storage of s-lysin we wanted to determine if it were stored intracellularly or in an extracellular site such as the periplasm or associated with the wall as had been reported by Millikin and Weiss (22) and Matsuda et al. (20). To do this we removed the walls from mt+ gametes with exogenously added lysin and then used immunoblot analysis with the anti-lysin antibody to determine if the de-walled cells still contained s-lysin. A control sample of cells was incubated in buffer instead of lysin. The results, shown in Fig. 10, indicated that whereas the control

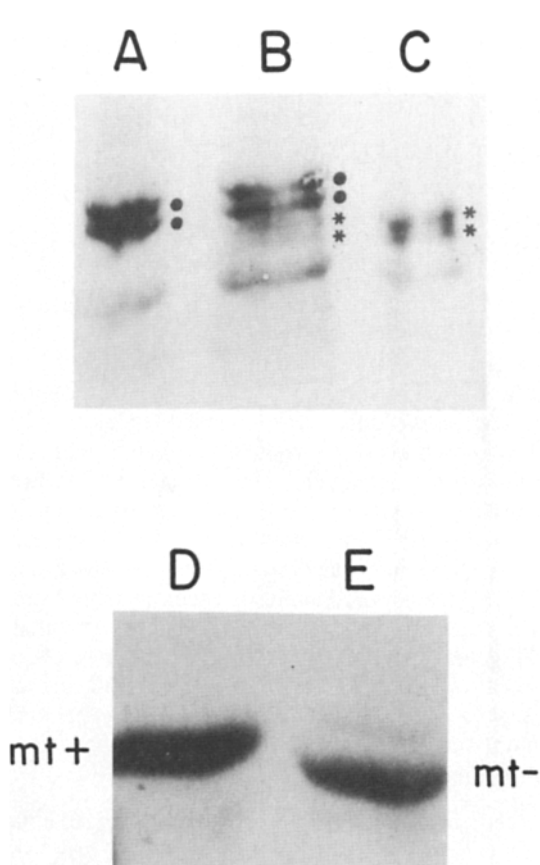


Figure 9. The s-lysins in mt+ gametes is of slightly higher relative molecular mass than that in mt- gametes. Suspensions of gametes mixed either before or after being boiled in SDS-PAGE sample buffer as described in the legend for Fig. 8 were subjected to electrophoresis on straight 10% gels and transferred to nitrocellulose paper. Lane A, the sample boiled before mixing, contained s-lysins. Lane C, the sample mixed before boiling, contained r-lysins. Lane B is a mixture of the two samples showing that both s-lysins (●) and r-lysins (*) were each doublet polypeptides. The lowest band in each of the lanes was a band of unknown origin and significance that sometimes appeared in these heavily loaded gels of intact cells. Mt+ and mt- gametes were also separately analyzed on these 10% gel immunoblots. Lane D is the mt+ gametes and lane E is the mt- gametes. The s-lysins in mt+ gametes was of slightly higher relative molecular mass than that in mt- gametes.

gametes contained s-lysins (Fig. 10, lane 2), the de-walled gametes did not (lane 1). Thus, s-lysins is stored outside of the plasma membrane.

We also determined that substantial amounts of s-lysins could be released from cells by freeze-thawing. To do this mt+ gametes were harvested from their medium as described above, washed into HC to a final concentration of 3×10^8 cells/ml, and frozen at -20°C . The sample was then thawed, frozen again, re-thawed, and centrifuged at $315,000\text{ g}$ ($85,000\text{ rpm}$; Beckman TL-100 Tabletop Ultracentrifuge, TLA-100.2 rotor; Beckman Instruments Inc., Palo Alto, CA) for 10 min. Immunoblot analysis of the supernatant showed that s-lysins was released in a soluble form by this treatment (Fig. 11, lane 1). Fig. 11, lane 2 shows a blot of r-lysins for comparison. About half of the cellular s-lysins was released by two cycles of freeze-thawing (data not shown). These results indicated that s-lysins was stored in a soluble form, probably in the

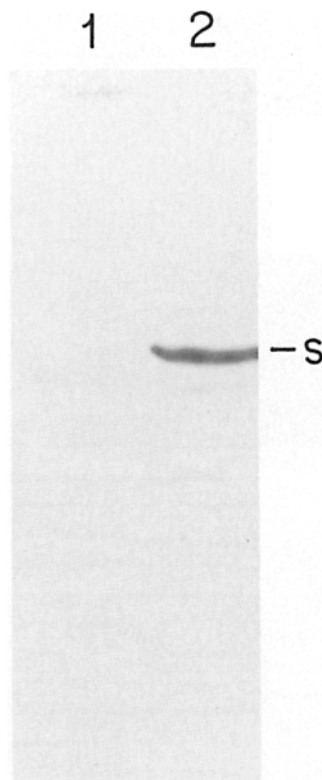


Figure 10. S-lysins is removed from cells when their walls are removed. Mt+ gametes were incubated with r-lysins until the wall loss assay showed the >90% of the cells had lost their walls. The suspension was then centrifuged and the sedimented cells were evaluated for the presence of s-lysins by immunoblotting with the anti-lysins antibody. A control sample of gametes was incubated with HC buffer for the same amount of time. Lane 1 shows the immunoblot of the de-walled gametes and lane 2 shows the control sample. Only the control gametes (lane 2) contained the antigen.

periplasm, and that freeze-thawing released it into the medium

We then wanted to learn more about the biological significance of the two forms of lysins. Because of the apparently intimate association of s-lysins with its substrate, we sus-

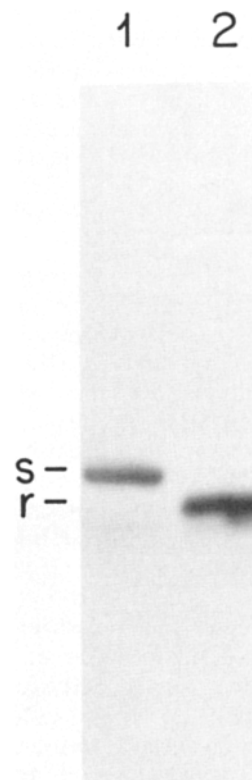


Figure 11. Freeze-thawing releases s-lysins from gametes. Mt+ gametes were frozen and thawed twice and then centrifuged as indicated in the text. The supernatant was evaluated by immunoblotting using the anti-lysins antibody (lane 1). Lane 2 shows a sample of r-lysins for comparison.

pected that s-lysin would be inactive. To determine this, samples of s-lysin prepared by the freeze-thawing method described above were evaluated in the standard lysin assay. This assay is sensitive enough to be able to detect r-lysin released from $\sim 1.5 \times 10^6$ mating gametes (4). Using several different preparations we have been unable to detect any lysin activity in s-lysin, even in assays that contained s-lysin prepared from 6×10^7 cells. These results indicate that s-lysin is stored as an inactive precursor, and during sexual signalling it is converted to r-lysin concomitant with activation.

Discussion

To learn more about the molecular mechanisms of sexual signalling and lysin release during the mating reaction in *Chlamydomonas reinhardtii* our laboratory has purified lysin and prepared a polyclonal antiserum against it. Antibodies directed against a common carbohydrate epitope found on several extracellular polypeptides were removed by absorption with cell walls, which are enriched in proteins that bear this epitope. The resulting antisera was shown by several criteria to be directed against the 60,000-*M_r* polypeptide that we (4) and others (15, 18) have previously shown is lysin. As expected, the antigen was released by gametes only during the mating reaction and was not detected in the medium of nonmating gametes. The antigen copurified with lysin activity in each of several different fractionation methods tested, including molecular sieve chromatography, velocity sedimentation in sucrose gradients, ion exchange chromatography, and chromatography on an affinity column prepared with the endogenous lysin substrate.

The result that mt+ and mt- gametes contained s-lysins of different relative molecular mass was somewhat unexpected. One explanation is that the difference is a consequence of strain-specific posttranslational modifications. For example van den Ende's group has shown that in *Chlamydomonas eugametos* there are strain differences in some *O*-methylated sugars on flagellar glycoproteins (12). The ability to *O*-methylate specific sugars in this species was inherited independently of mating type and presumably is due to different methyltransferase alleles (12). Future experiments should help to establish if the difference in relative molecular mass of s-lysins we show here for *Chlamydomonas reinhardtii* is linked to mating type.

The availability of the antibody made it possible to learn more about the cellular mechanisms for storage and release of lysin during the mating reaction. Immunoblot analysis of unmated gametes showed that the stored form of lysin, s-lysin, was of slightly higher relative molecular mass than the released form of lysin, r-lysin. S-lysin was 62,000 *M_r*, whereas, r-lysin was 60,000 *M_r*. In experiments to identify the cellular site for storage of s-lysin, we found that s-lysin was missing in cells whose walls had been removed by treatment with exogenously added r-lysin. This result, coupled with the fact that s-lysin could be recovered in a 315,000 g supernatant from frozen and thawed mt+ gametes, was consistent with the idea that s-lysin is stored in the periplasm of gametes. Future immunolocalization experiments at the electron microscopic level will be important to identify directly the storage site for s-lysin.

The result that this wall degrading enzyme appeared to be stored in such close proximity to its endogenous substrate suggested that the storage form of the enzyme might be inac-

tive. We were able to test this possibility by assaying preparations of s-lysin for lysin activity. We found that preparations of s-lysin, obtained by freeze-thawing cells, contained no lysin activity detectable in our standard lysin assay. Only r-lysin was active. Thus, lysin is stored as an inactive proenzyme that is converted to the active enzyme as a consequence of sexual signalling.

Although they did not interpret their data in this way, other *Chlamydomonas* workers have presented evidence that is consistent with the idea that the stored enzyme is inactive. Claes (7) reported that the lysin present in cells disrupted by freezing and thawing was inactive and could be detected only after sonication. Recently, Matsuda et al. (20) obtained similar results. They showed that only extremely low levels of lysin could be detected in cells disrupted by freezing and thawing, even though that treatment released another enzyme, acid phosphatase. These workers found that sonication or homogenization by the French press was required to yield an active enzyme. The interpretation suggested by this group was that the enzyme is stored either in a sedimentable form, for example, a membrane-bounded vesicle or bound to an inhibitor. Consistent with this observation was the result of Matsuda et al. (20) that the form isolated from freeze-thawed, French-pressed gametes and the form that appeared in the medium during mating were indistinguishable on their 7.5–15.0% gradient SDS-PAGE gels.

The results presented here suggest an alternative explanation for the observation that the stored form of the enzyme cannot be detected without homogenization or sonication. As indicated above, the 62,000-*M_r* precursor is inactive until it is converted to the 60,000-*M_r* form. We would propose that this conversion is a consequence of sexual signalling. Possibly a separate converting enzyme is activated or secreted through the action of one of the second messengers, such as Ca^{2+} (3) or cAMP (29, 30), reported to appear during signalling. Since Matsuda apparently isolated the 60,000-*M_r* form from homogenized cells, the process of homogenization or sonication might lead to the conversion that normally accompanies mating. For example, homogenization or sonication could activate the molecule that normally is activated as a consequence of signalling, or at least homogenization could permit the coming together of the putative converting enzyme and s-lysin. The idea that there is a molecule, possibly a protease, that converts s-lysin to r-lysin is currently being tested in our laboratory.

There are several noteworthy aspects of a *Chlamydomonas* periplasmic zymogen that is activated as a consequence of cell contact. First, the lysin activation that we have described has many striking functional similarities to the proacrosin-acrosin system in mammalian sperm (28). Acrosin, a protease that is thought to be required for penetration of the sperm through the outer vestments of the egg is stored as inactive proacrosin in the acrosome. Interactions between the sperm and egg during fertilization induce the acrosome reaction, leading to conversion of proacrosin to acrosin. Future experiments should reveal if there are molecular similarities underlying these functionally analogous processes. Second, to our knowledge there are only a few examples of zymogens in lower eucaryotes or in organisms containing chloroplasts (24, 26). Thus, studies on lysin activation might yield new information about the evolution of proenzymes and their processing.

Finally, it is possible that lysin plays a somewhat different

role in vegetative cells compared to gametes. Matsuda et al. (20) have reported that vegetative cells also contain lysin, and preliminary, unpublished immunoblotting experiments in our laboratory have shown that vegetative cells contain the lysin antigen. Although, the function and location of lysin in vegetative cells are unknown, it is likely that the enzyme plays some role in expansion of the cell wall during vegetative growth. Cells undergo dramatic changes in size during growth, and unless the wall is flexible, it is likely that growth of the wall occurs by localized lysis followed by insertion of new wall components as has been suggested for bacterial wall growth (38). Moreover, Matsuda et al. have reported that lysin is stored in different cellular compartments in vegetative cells and gametes (19). It will be interesting to learn how intracellular targeting and activation of this molecule is regulated as vegetative cells differentiate into gametes.

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