Purification and characterization of cell wall lytic enzyme released by mating gametes of *Chlamydomonas reinhardtii*

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A cell wall lytic enzyme of *Chlamydomonas reinhardtii* has been purified and identified as a single glycopolypeptide subunit of 62 kDa by SDS–polyacrylamide gel electrophoresis. It is released into culture medium by mating gametes as a large aggregate of subunits. The purified enzyme shows a pH optimum at about 7.5 and 35°C. Metal ion chelators and SH-blocking agents inhibit the activity. The activity is also diminished by α_2-macroglobulin.

Chlamydomonas reinhardtii *Cell wall* *Lytic enzyme* *Gamete* *Mating* *Glycopolypeptide*

1. INTRODUCTION

Gametes of the unicellular green alga *Chlamydomonas reinhardtii* shed their cell walls during mating in preparation for cell fusion [1,2]. This shedding is caused by the activity of cell wall lytic enzyme that has been induced in cells by the signal of flagellar agglutination between two mating-type gametes, mating-type plus (mt^+) and minus (mt^-) [3,4]. The lytic enzyme is generated mainly from the mt^+ gametes and excreted into culture medium where it acts on other walled gametes from the outside [2,5,6].

The lytic enzyme totally dissolves the cell walls at all cell stages (vegetative and gametic cells and zoosporangia) except the walls of the zygospore [7]. Therefore, the crude enzyme has been used successfully to prepare protoplasts for the study of cell wall formation [8], genetic analysis by polyethylene glycol-induced somatic cell fusion [9,10] and measurement of cellular activity [11].

In [12], we developed procedures for the convenient quantitative assay of the lytic activity and for partial purification of the enzyme under stabilized conditions. We report here the purification, identification and some properties of the lytic enzyme.

Abbreviations: Con A, concanavalin A; DEP, diethyl pyrocarbonate; PCMB, *p*-chloromercuribenzoic acid; PMSF, phenylmethylsulphonyl fluoride

2. EXPERIMENTAL

2.1. *Preparation of crude enzyme*

Gametes of the wild-type strain 137c, mt^+ and mt^-, were prepared as in [13]. About 700-ml cultures of mt^+ gametes were mixed with equal volumes of mt^- gametes at a density of about 4×10^7 cells/ml at 25°C. After 15 min, 350 ml of 50 mM Tris–acetate (pH 7.5), 1 M NaCl was added to the mixed gamete suspension to stop the mating reaction and to stabilize the lytic enzyme [12]. The cell-free supernatant was prepared by centrifugation at $7500 \times g$ for 10 min at 4°C and used as the source of the enzyme in the purification procedure.

2.2. *Purification procedure*

The crude enzyme was precipitated by ammonium sulfate at 60% saturation. After centrifuging at $13000 \times g$ for 20 min, the pellet was resuspended in 50 ml of 10 mM Tris–acetate (pH 7.5), 200 mM NaCl, 2 mM dithiothreitol (solution A) and dialyzed against solution A overnight at

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4°C. The enzyme solution was clarified at 100000 × *g* for 30 min and then applied to Con A–Sepharose 4B gel (Pharmacia; 1 × 16.5 cm, 7 ml) at a flow rate of 4.5 ml/h. After extensive washing of the column with solution A, elution of enzyme was performed with 0.1 M methyl-α-D-mannoside in solution A. The peak fractions of enzyme were passed through a Toyopearl HW-50 column (gel for high-performance gel filtration, 2 × 50 cm; Toyo Soda Co.) at a flow rate of 40 ml/h and then the eluate was adsorbed onto hydroxyapatite (Biogel HTP, 2 ml) at a flow rate of 4.5 ml/h. After washing the column with solution A, elution was carried out with a linear gradient of 0–0.1 M sodium phosphate buffer (20 ml), pH 7.0. The purified enzyme thus obtained was dialyzed against solution A and kept frozen at −70°C until use. In some experiments, the enzyme solutions were concentrated or the buffer composition exchanged by use of Minicon-A (Amicon). Protein contents were determined by the Folin method [14] with bovine serum albumin as a standard.

2.3. *Enzyme assay*

The assay procedures of enzyme activity were essentially as in [12]. However, the zoosporangia used as substrates were fixed with 0.1% glutaraldehyde rather than 0.25%. The reaction mixtures contained 10 mM Tris–acetate (pH 7.5), 0.5 mg/ml bovine serum albumin, 1 mM $MgCl_2$, 2.5×10^5 fixed zoosporangia and 0–20 μl enzyme solution in a total volume of 0.25 ml. The reaction was run at 35°C rather than 25°C [12] for 30 min and stopped by the addition of EDTA (20 mM final concentration). One unit of enzyme is defined as the amount which liberates daughter cells from 1×10^6 zoosporangia at the 50% level [12].

2.4. *Gel electrophoresis*

SDS–polyacrylamide slab gel electrophoresis was carried out in a discontinuous buffer system [15] using a 4–15% linear acrylamide gradient with 3.5% stacking gel. Materials were dissolved in sample buffer (10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.004% bromophenol blue, 70 mM Tris–HCl, pH 6.8) and heated for 5 min in a boiling water bath. Pharmacia high-M_r calibration kit was used for protein standards. The gels were run at a constant 12 mA for 5–6 h at 15°C. Non-SDS gradient gel electrophoresis for native proteins was performed using the discontinuous buffer system without SDS or the standard Tris–boric acid–EDTA buffer according to the instruction manual for Pharmacia calibration proteins. In some experiments non-SDS gel electrophoresis was carried out under a high salt condition: NaCl (0.5 M) was added to the gel and upper electrode buffer. After gel electrophoresis, proteins were stained with Coomassie blue or with silver stain [16]. Carbohydrates were stained by a rapid method (in preparation) based on a periodic acid-silver stain procedure as in [17].

3. RESULTS

3.1. *Enzyme purification*

The lytic enzyme is known to be precipitated with 50–75% ammonium sulfate [7], to become

Table 1

Purification of cell wall lytic enzyme released by mating gametes

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification factor	Percent recovery
Mating medium	1420	136.3	2.2×10^5	1.6×10^3	1	100
Ammonium sulfate (60%)	114	70.1	2.0×10^5	2.9×10^3	2	91
Con A affinity chromatography	11	1.7	9.1×10^4	5.4×10^4	34	41
Gel filtration	30	0.9	7.4×10^4	8.2×10^4	52	34
Hydroxyapatite chromatography	7	0.2	4.4×10^4	2.2×10^5	138	20

uniform in size in the presence of 0.2 M NaCl [12] and to bind specifically to a Con A–agarose column [18]. These properties served as a basis for the purification of the enzyme as described in section 2. Con A affinity chromatography of the crude enzyme resulted in increase of the specific activity by about 20-fold (table 1). However, the enzyme fraction still contained many protein impurities (fig.1, lane 1). Therefore, we further purified the enzyme by gel filtration and hydroxyapatite chromatography and finally identified it as a single glycopolypeptide band in SDS-polyacrylamide gels after staining with Coomassie blue (not shown), silver (fig.1, lane 2) and periodic acid-silver (lane 4). The molecular mass was 62 kDa. The final purification was about 140-fold and the recovery 20% (table 1).

We have shown that the lytic enzyme is found as large aggregates with diverse molecular masses in culture medium or under low concentrations of salt [12]. This was confirmed by the analysis on non-SDS gel electrophoresis (fig.2). The purified enzyme migrated only into the stacking gel, presumably due to the heavy aggregation, under the normal condition of gel electrophoresis (fig.2, lane 1). When the non-SDS gel electrophoresis was carried out in 0.5 M NaCl, the enzyme moved into the separating gel and was observed as several broad bands with approximate molecular masses of 120, 180, 220 kDa and higher (fig.2, lane 2). These may represent small aggregates (dimer, trimer, tetramer, etc.) of enzyme subunits.

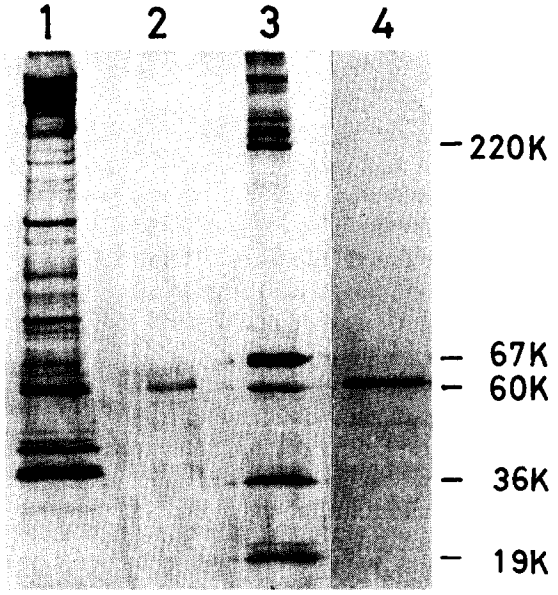

Fig.1. SDS–polyacrylamide gel electrophoresis of lytic enzyme. Samples were analyzed in a 4–15% acrylamide gradient SDS gel and by silver (lanes 1–3) or periodic acid-silver (lane 4) staining. Lane 1, enzyme fraction after Con A affinity chromatography; lanes 2 and 4, purified enzyme after gel filtration and hydroxyapatite chromatography; lane 3, marker proteins: ferritin half unit (220 kDa), bovine serum albumin (67 kDa), catalase (60 kDa), lactate dehydrogenase (36 kDa) and ferritin (19 kDa).

3.2. *Characterization of enzyme*

The purified enzyme had a pH optimum at ~7.5 and temperature ~35°C. The activity was lost completely within 5 min incubation at 50°C. Divalent cations (Ca^{2+}, Mg^{2+}) enhanced the lytic activity by 10–40%, the optimum concentration being 0.75–1 mM. The presence of 2 mM dithiothreitol stabilized the enzyme further.

Table 2 lists the results of inactivation experiments with various inhibitors. Chelators of the metal ions (EDTA, EGTA, 1,10-phenanthroline) and SH-blocking agents (PCMB, DEP, $HgCl_2$,

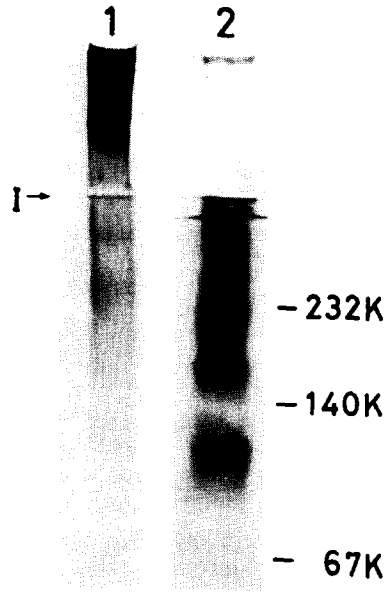

Fig.2. Non-SDS gradient gel electrophoresis of purified enzyme. The purified enzyme was analyzed in non-SDS gels in the standard buffer (lane 1) or in the buffer containing 0.5 M NaCl (lane 2) and by silver staining. I, stacking gel interface.

Table 2

Effect of inhibitors on lytic activity

Inhibitor	Concentration (mM)	Percent inhibition
EDTA	1	81
	2	95
EGTA	1	2
	2	56
PCMB	1	85
	2	100
$HgCl_2$	0.04	5
	0.1	100
DEP	1	36
	2	100
Iodoacetate	2	16
	3	92
PMSF	2	4
Trypsin inhibitor	1 mg/ml	3
ϵ-Aminocaproic acid	30	0
Pepstatin A	0.1 mg/ml	5
1,10-Phenanthroline	0.02	66
	0.04	100
α_2-Macroglobulin	0.2 mg/ml	26
	0.4 mg/ml	100

Inhibitor was preincubated with lytic enzyme in 0.24 ml reaction mixture for 15 min at 35°C, then substrate (2.5×10^5 zoosporangia, 10 μl) was added and incubation continued for 30 min. $MgCl_2$ was omitted from the reaction mixture

iodoacetate) inhibited the activity. A widespread protease inhibitor, α_2-macroglobulin (Boehringer-Mannheim) also diminished the activity. PMSF, soybean trypsin inhibitor, ϵ-aminocaproic acid and pepstatin A, however, had no effect.

It has been reported that in its crude form, the purified enzyme dissolves vegetative and gametic cell walls as well as the mother (sporangial) cell walls of *C. reinhardtii*, but is unable to digest those of other species such as *C. eugametos* and *C. moewusii*. For the complete release of daughter cells from 1×10^6 zoosporangia, only about 2 units of enzyme were required, whereas 40–80 units were necessary to digest the cell walls of 1×10^6 living vegetative cells. When cell walls were isolated as in [19] and treated with the enzyme, at least 300 units were required for 1 mg/ml (about 5×10^6 cell walls) wall solution until they had become completely invisible by phase contrast microscopy.

4. DISCUSSION

Besides the cell wall lytic enzyme released by mating gametes, which has been purified and characterized in the present study, a second-type lytic enzyme, 'sporangial wall-autolysin', or hatching enzyme which is excreted into the medium by synchronously dividing cells on daughter cell liberation, is known in *Chlamydomonas* [20,21]. It has been reported [7] that although the hatching enzyme can only lyse the sporangial walls, it has some characteristics in common with the lytic enzyme released by mating gametes. Authors in [22] have purified the hatching enzyme and characterized it as a serine protease. Comparison of their data [22] with those presented here indicates that the two enzymes are distinctly different:

(i) The hatching enzyme exists as a single (glyco)protein molecule of 37–40 kDa in its native and reduced form, whereas the lytic enzyme consists of a glycopolypeptide subunit of 62 kDa and occurs natively as aggregates.

(ii) Chelators of the metal ions and SH-blocking agents do not inactivate the hatching enzyme, but do inactivate the lytic enzyme.

(iii) The hatching enzyme can digest proteins such as casein and insulin. It is inactivated by inhibitors of serine protease and of trypsin. The lytic enzyme, however, appears to be insensitive to such inhibitors. Our preliminary results showed that there is no significant digestion of casein and insulin by the lytic enzyme.

It is not known whether the cell wall lytic enzyme released by mating gametes acts on the carbohydrates or proteins of *Chlamydomonas* cell wall [6]. However, since this enzyme is inactivated by α_2-macroglobulin, chelators of the metal ion and SH-blocking agents, it may be a metal and/or thiol protease (cf. [23]).

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