IDENTIFICATION AND PARTIAL PURIFICATION OF A LYTIC ENZYME IN THE BACTERIOPHAGE ϕ6 VIRION

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1. Introduction

Bacteriophage ϕ6 of *Pseudomonas phaseolicola* is surrounded by a membrane-like structure and contains three segments of double-stranded RNA as its genetic material [1–3]. Electron microscopic study indicated that, in the early step of infection, the membrane of ϕ6 and the bacterial outer membrane fuse to form a bulge and the nucleocapsid penetrates the cytoplasmic membrane [4,5]. These observations are also supported by biochemical experiments in our laboratory (Kakitani, H. I., Y. O., in preparation). We found a lytic enzyme associated with ϕ6 virion [6] and suggested that the enzyme might be necessary for the penetration of nucleocapsid through the peptidoglycan layer of the host cell.

In this study, the lytic enzyme was solubilized from ϕ6 virion, partially purified, and identified as protein P5 (mol. wt 26 000). The small protein, P10 (mol. wt ~6000) is also suggested to be a possible component of the lytic enzyme.

2. Materials and methods

2.1. *Bacterium and phage*

Bacteriophage ϕ6 and *Pseudomonas phaseolicola* HB10Y, orginally obtained from Dr A. K. Vidaver, were used. The bacterium was grown in MSC medium [7] at 25°C and ϕ6 was purified from the lysate as in [6]. The purified ϕ6 was finally suspended in 35 mM *N,N*-bis(2-hydroxyethyl)glycine (Bicine) (pH 7.8) ($2.3–10 \times 10^{12}$ p.f.u./ml).

[^{3}H]Leucine-labelled ϕ6 was prepared from the lysate of M8 medium [8] ([^{3}H]leucine, 10 μCi/ml) by the same procedure.

2.2. *Assay of the lytic enzyme*

A culture of *P. phaseolicola* (2 l) was grown in M8 medium (2.5×10^8 c.f.u./ml), washed once by a low speed centrifugation, suspended in 40 ml ice cold water, and added to 3 ml chloroform. The mixture was gently shaken for 2 h at 0°C. Chloroform phase was discarded and the remaining chloroform was evaporated by bubbling of N_2 gas. The cell suspension was frozen in small fractions, stored at –70°C, and diluted with 0.1 M Tris–HCl (pH 7.2) to give an A_{450} of 1.0 (Gilford spectrophotometer model 250) immediately before use. This substrate cell suspension (1 ml each) was mixed with 20 μl (or 50 μl) of the enzyme sample or the same volume of the buffer alone at 20°C. The A_{450} of both sample mixture and control were monitored and the difference between them was measured every 1 min to determine the initial rate of decrease in A. One lytic unit (LU) was defined as a decrease in A_{450} of 0.01/min.

2.3. *SDS–polyacrylamide gel electrophoresis*

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was carried out as described by Laemmli [9]. The protein sample was added to the gel sample buffer (final conc. : 0.0625 M Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue) and boiled at 100°C for 2 min. Usually 15% slab gel was used and stained with Coomassie brilliant blue.

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3. Results

3.1. *Solubilization of the lytic enzyme from φ6 virion*

To solubilize the lytic enzyme from virion, we treated φ6 with sodium deoxycholate (NaDOC) which caused no lysis of chloroform-treated *P. phaseolicola* cells and did not interfere with the assay of lytic activity. Lytic activity drastically increased after 0.1% NaDOC treatment of φ6 virion which accompanied a decrease in infectivity (fig.1). At 0.2% NaDOC, the infectivity was lost, and a partial disruption of nucleocapsid began to occur, interfering the further purification of the lytic enzyme (data not shown). We, therefore, treated φ6 with 0.15% NaDOC for the solubilization of the lytic enzyme.

φ6 solution (1.0×10^{13} p.f.u./ml) containing [^{3}H]Leu-labelled φ6 was gently mixed with an equal volume of 0.3% NaDOC, 35 mM Bicine (pH 7.8) and 20 mM 2-mercaptoethanol at 0°C. After 10 min, the mixture was centrifuged at 60 000 × *g* for 50 min. All of the lytic activity was recovered to the supernatant fraction (table 1). Protein component of the supernatant and the pellet were analyzed by SDS–polyacrylamide gel electrophoresis (fig.3A). The supernatant fraction contained P3, P5, P6, P9 and P10, which were reported to be membrane components [3]. The pellet fraction contained nucleocapsid proteins P1, P2, P4, P7 and P8 [10] with small amount of contaminated proteins P3 and P9. From these results, the lytic enzyme is shown to be included in the membrane protein of φ6.

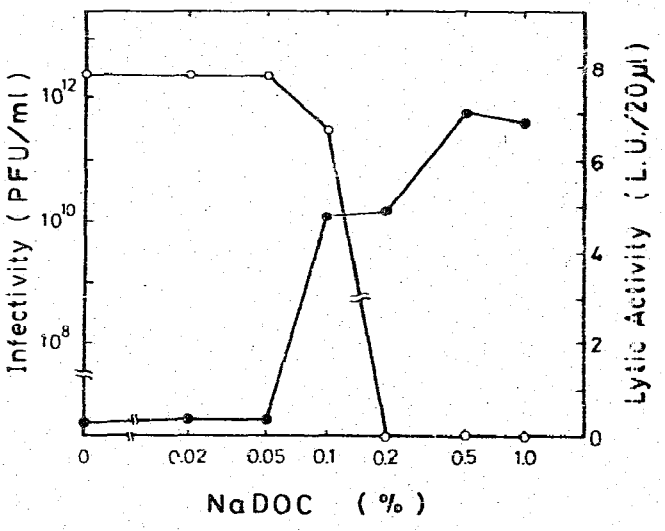

Fig.1. Disruption of φ6 particle by the treatment of NaDOC. Purified φ6 (0.1 ml each) was gently mixed at 0°C with the same volume of 35 mM Bicine (pH 7.8) and 20 mM 2-mercaptoethanol containing various concentrations of NaDOC. After 10 min, infectivity (○) and lytic activity (●) were assayed.

3.2. *Partial purification and identification of the lytic enzyme*

The supernatant fraction was placed on a Sephadex

Table 1
Purification of lytic enzyme from φ6 particle

Determinations	φ6 particle	φ6 treated with 0.15% NaDOC	Supernatant fraction	Peak fraction[a] of Sephadex G-200
Total volume (ml)	2.1	4.2	3.8	10.0
Total lytic activity (LU)	272	2780	2550	556
Total protein ([^{3}H]Leu cpm)	4.7×10^5[b]	4.7×10^5	9.6×10^4	1.4×10^4
Spec. act. (LU/^{3}H cpm)	5.8×10^{-4}	5.9×10^{-3}	2.6×10^{-2}	4.0×10^{-2}
Yield of lytic activity (%)[c]	–	100	92	20
Purification[c] (-fold)	–	1	4.5	6.8

[a] Fractions 37–41 were taken as the peak fraction (fig.2)
[b] It corresponds to ~2.5 mg protein
[c] Based on the lytic activity of disrupted φ6 particle

G-200 column (1.4 X 70 cm) equilibrated with 0.15% NaDOC, 35 mM Bicine (pH 8.5) and 10 mM 2-mercaptoethanol and eluted with the same buffer at 0°C. Two radioactive (protein) peaks were observed, and the lytic activity recovered as a single sharp peak near the end of the second protein peak (fig.2).

Each fraction was analyzed by SDS–polyacrylamide gel electrophoresis (fig.3B). The lytic activity was completely accompanied with protein P5, and protein P5 was not found in other fractions. It is concluded that protein P5 is a component of the lytic enzyme.

Though protein P10 could not be stained well owing to its small molecular weight (~6000), the protein was shown to also accompany the lytic activity when a large amount of protein was applied in SDS–polyacrylamide gel electrophoresis (fig.3B,

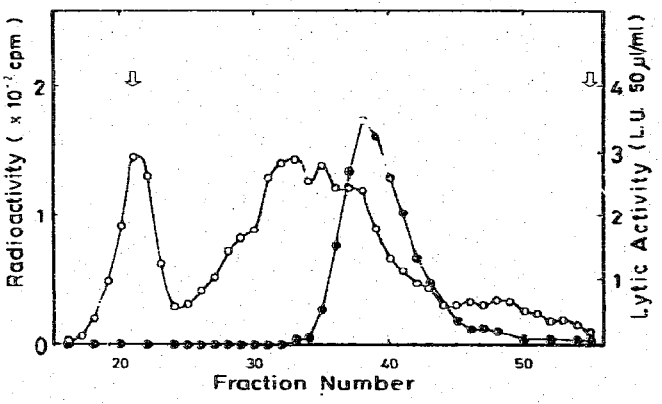

Fig.2. Gel filtration of φ6 membrane proteins on Sephadex G-200. From each fraction (2 ml), 0.1 ml and 0.05 ml aliquots were withdrawn for the assay of radioactivity (○) and lytic activity (●), respectively. Arrows indicate void and total volumes.

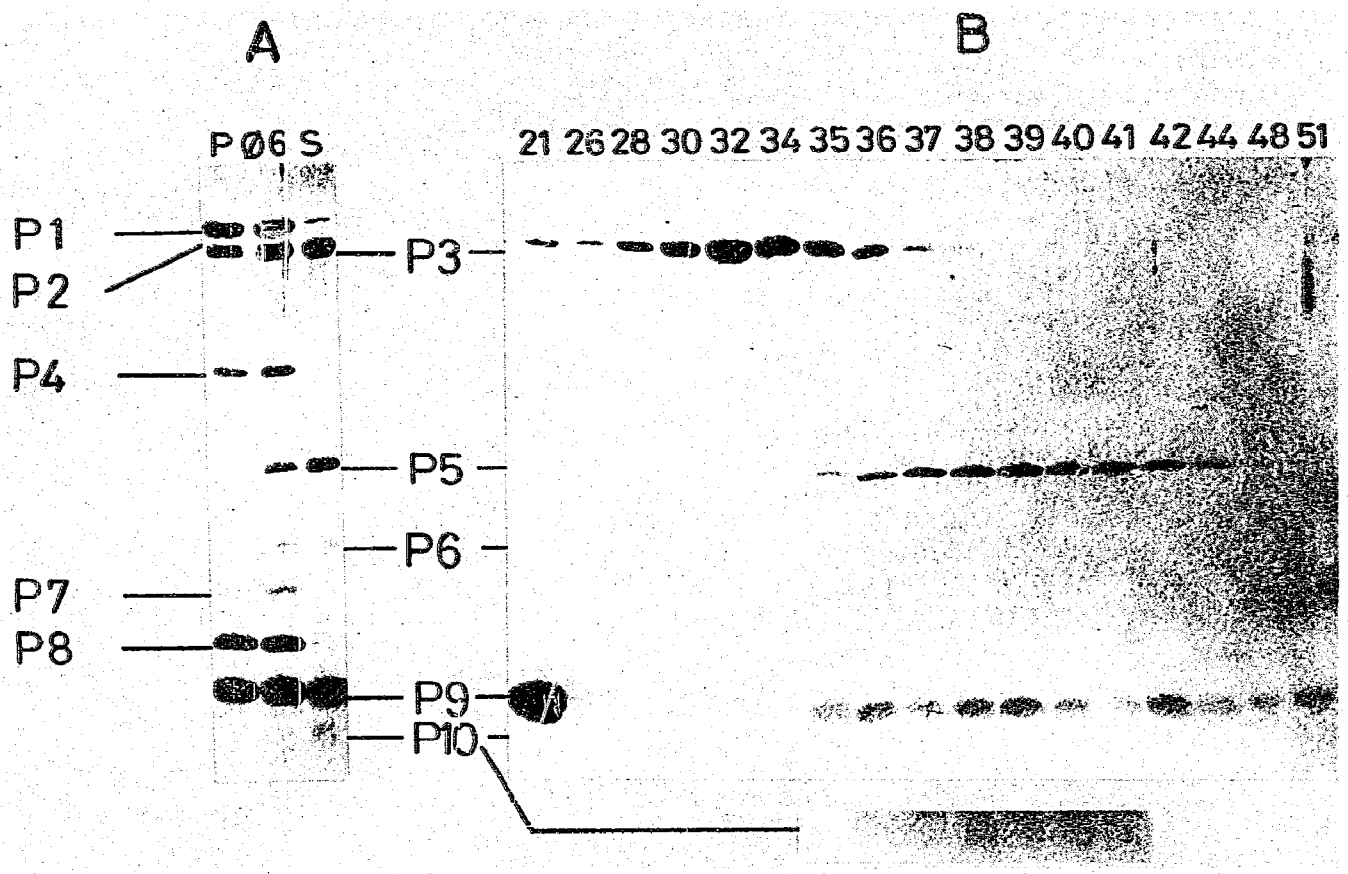

Fig.3. SDS–polyacrylamide gel electrophoresis of protein samples in the purification steps of the lytic enzyme. (A) The supernatant (S) and the pellet (P) of φ6 particle treated with 0.15% NaDOC and the intact φ6 particle (φ6) were analyzed. (B) Fractions (1.8 ml each) of Sephadex G-200 chromatography (fig.2) were added with trichloroacetic acid (final 10%). The precipitate was collected by a low speed centrifugation and washed twice with acetone. One-fourth of the protein samples were analyzed on a slab gel. Numbers show the fraction number in fig.2. The lower strip shows protein P10 region of another gel electrophoresis applied by a 3-fold amount of protein samples.

lower strip) (see section 4). There is also a faint band of protein P9 in the peak fraction of the lytic activity but most of the protein P9 eluted at the void volume. Protein P6, a minor membrane protein was shown to elute only at the void volume by another gel electrophoresis of a large amount of protein samples (data not shown).

3.3. *Stability of the lytic enzyme*

The lytic enzyme was unstable even at 0°C when it was solubilized. EDTA (1 mM), 10% sucrose, and phenylmethyl sulfonyl fluoride (200 μg/ml) failed to prevent the loss of enzyme activity, but the addition of 10 mM 2-mercaptoethanol partly stabilized the activity. The overall recovery of the lytic activity from NaDOC-treated virion was 20% with a 6.8-fold purification (table 1) in the presence of this reagent.

4. Discussion

The lytic activity associated with φ6 particle [6], has been solubilized and partially purified from the virion. A membrane protein P5 was shown to completely accompany the lytic activity on Sephadex G-200 column chromatography indicating that protein P5 is a component of the lytic enzyme. Though protein P10 was difficult to stain after gel electrophoresis, this protein, as well as protein P5, was detected only in the fractions that had the lytic activity (fig.3B). Thus this small protein is suggested to be a possible component of the lytic enzyme. Separation of proteins P5 and P10 would be needed to confirm this possibility. Partially purified enzyme, however, was very unstable (60% loss of activity during 8 h storage at 0°C) and the further purification of the enzyme was unsuccessful at this time. We can not completely exclude the possibility that a small amount of protein P9 existing in the purified enzyme fraction might be necessary for the lytic activity.

Purified φ6 had only a little amount of lytic activity, but it was drastically activated by the phage disruption (fig.1). This result suggests that the activity is repressed in the intact virion. As the lytic enzyme is a component of φ6 membrane, the enzyme could be expected to function when the membrane of this phage fuses with the outer membrane of the host cell in the early process of infection, allowing the nucleocapsid to penetrate the cytoplasmic membrane.

Acknowledgement

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