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ENZYMATIC ACTIVITIES ON CELL WALLS IN BACTERIOPHAGE T4

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SUMMARY

When isolated particles of bacteriophage T4 are disrupted by freezing and thawing, they exhibit a lytic activity (particle-bound lysozyme). T4*e am* phage, which has an *amber* mutation in the structural gene for phage lysozyme, propagated in the restrictive host is devoid of this lytic activity. In addition, a temperature-sensitive mutant in phage lysozyme gene exhibits the particle-bound lytic activity which is also temperature sensitive. It follows from these facts that particle-bound lysozyme and phage lysozyme found free in the lysate are directed by the same structural gene, gene *e*. The particle-bound lysozyme is not essential to phage infection nor to 'lysis-from-without', although these events were postulated to be enzymatic.

An enzymatic activity which attacks the cell walls of *Escherichia coli* and which is different from that of phage lysozyme was found in isolated T4 phage. It differs from the phage lysozyme in the following respects. (1) The optimum pH of the enzyme lies between 3.5 and 5, while that of the phage lysozyme is around 7. (2) The enzyme lyses neither chloroform-treated cells of *Escherichia coli* nor intact cells of *Micrococcus lysodeikticus*. (3) The enzyme is found in T4*e am* phage, which is deficient in the phage lysozyme.

INTRODUCTION

T-even phages carry on their tail structure an enzyme which splits a component from isolated cell walls[1,2]. Since the discovery of this enzymatic activity on phage particles, it has been postulated that this enzyme may play some role in the penetration of phage components through the wall of the host cell[1,3]. It was also shown that this enzyme has the properties of lysozyme[4].

On the other hand, T-even phage lysates contain large quantities of lytic enzyme in soluble form. The formation of the lytic enzyme is controlled by the phage genome, and T4 phage mutants unable to synthesize the lytic enzyme can not lyse the host cell[5]. The lytic enzyme digests the infected cell from within and liberates the progeny phage from the host bacterium. The enzyme was characterized as a lysozyme[6,7], and is now called phage lysozyme.

Although the properties of phage lysozyme found free in lysates closely resemble

those of particle-bound lysozyme, no definite conclusion has been reached concerning the identity of these two lysozyme activities. It remains to be determined whether particle-bound lysozyme is different from free lysozyme and whether it really forms an integral part of, or is only casually adsorbed to, the phage particle. It has become possible to answer this question by experiments with phage mutants in the *e* gene, isolated first by STREISINGER *et al.*[5].

This paper will describe (1) the identity of particle-bound lysozyme and free lysozyme, (2) some functional aspects of particle-bound lysozyme and (3) the presence of an enzymatic activity different from that of lysozyme in isolated T4 phage.

A preliminary account of a part of this work has been reported[8].

MATERIALS AND METHODS

Bacterial strains. Escherichia coli B was used in most of these experiments. *E. coli* CR63, a permissive host, was used for the propagation of T4 *amber* mutants.

Bacteriophage strains. All phage strains were from the collection of Dr. M. SEKIGUCHI. T4B*e*45 is a mutant that produces temperature-sensitive lysozyme[5]. T4B*e*HS90 and T4D*e*H26 are *amber* mutants in the lysozyme gene, which were originally isolated by G. STREISINGER and by R. S. EDGAR, respectively. T4D*am*N93 and T4D*am*N69 are *amber* mutants in genes 11 and 12 (ref. 9).

Media and growth of microorganisms. Bacteria were grown in M9 medium supplemented with Casamino acids (2 mg/ml) at 37° under vigorous aeration, and L-tryptophan (20 μg/ml) was added just before infection to improve phage adsorption. The preparation and assay of phages were those described by ADAMS[10].

Preparation of ^{14}C-labelled cell walls. E. coli B was grown in a synthetic medium[2] containing uniformly ^{14}C-labelled glucose (20 μC/mg; 1 μg/ml) at 37° for at least 12 h. The cells were disrupted in a 20-kcycle sonic oscillator (Measuring and Scientific Equipment) for 2 min, and the cell walls were isolated by the procedure of BROWN AND KOZLOFF[2].

Procedures to liberate T4e am phage from the restrictive host. Two procedures were used. *Autolysis*: T4*e am*-infected cells are centrifuged down, suspended in the original volume of Tris–HCl buffer (10 mM, pH 7.5), and incubated overnight at 37° in the presence of 0.01 vol. of chloroform. *Lysozyme treatment*: The infected cells are washed once with water, resuspended in 0.1 the original volume of Tris–HCl buffer (50 mM, pH 7.5) saturated with chloroform, and then kept at 25° overnight with egg white lysozyme (final concentration: 0.3 μg/ml).

Isolation and alteration of phage particles. After removal of unlysed cells and debris by low-speed centrifugation (3000 $\times g$, 20 min), the lysate was centrifuged at 20 000 $\times g$ for 60 min. The sedimented phage particles were resuspended in 0.1 the original volume of M9 medium and kept at 25° overnight. This washing cycle was repeated twice more, and phages were finally suspended in water. Pancreatic deoxyribonuclease (10 μg/ml) and 5 mM Mg^{2+} were added to the phage suspension, and frozen

and thawed 5 times alternately in an acetone–dry-ice bath and in a 37° water bath unless otherwise noted. This treatment inactivated over 99% of the virus.

Assay of enzymes. Lysozyme activity was measured by the method described by SEKIGUCHI AND COHEN[11]. A Klett–Summerson photoelectric colorimeter (540 mμ) was used for the turbidimetry measurement.

The breakdown of ^{14}C-labelled cell wall material was measured by suction filtration of the incubation mixture through a membrane filter (Millipore filter PH). About 0.5% of the radioactivity in the cell wall passes through the filter after the incubation in the absence of enzyme, and this value was subtracted from the amount of ^{14}C found in the filtrate after the enzyme–cell wall interaction.

RESULTS

Absence of lytic activity in isolated particles of T4e am phage propagated in the restrictive host

Chloroform-treated cells of *E. coli* are lysed by phage lysozyme as well as by egg white lysozyme, and can be used as substrate for the assay of phage lysozyme[11]. It was found that T4 phage particles altered by freezing and thawing exhibit a considerable lytic activity, while intact particles do not lyse the chloroform-treated cells, as shown in Fig. 1A. Under the same conditions, a phage-free lysate, which contains large quantities of free lysozyme, exhibits a strong lytic activity.

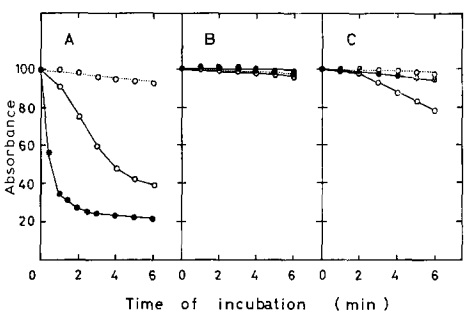


Fig. 1. The lytic activities of lysates and altered phage. T4*e*HS90 propagated in the restrictive host was liberated from the host cell by the autolysis method. The preparation of altered phage and the assay of lytic activity are described in METHODS. The amount and type of phage added to the incubation mixtures (4 ml total vol.) were: (A) $1 \cdot 10^{11}$ T4e^+ propagated in *E. coli* B; (B) $1 \cdot 10^{12}$ T4*e*HS90 propagated in *E. coli* B; (C) $4 \cdot 10^{11}$ T4*e*HS90 propagated in *E. coli* CR63. For the assay of the lytic activity of lysates, 0.2 ml of phage-free lysate (corresponding to approx. $1 \cdot 10^{10}$ phage) was used. ○ · · · · ○, intact phage; ○—○, altered phage; ●—●, phage-free lysate.

In order to determine whether the lytic activity in isolated phage particles is due to the phage lysozyme adsorbed to phage particles or due to another enzyme, the lytic activity of isolated T4*e am* phage was examined. If the particle-bound lytic activity is directed by a structural gene different from that for free lysozyme (*e* gene), the lytic activity will be detected in T4*e am* phage propagated in the restrictive host. And if it is directed by the same gene, these particles will be devoid of the activity.

Fig. 1B shows that no significant lytic activity is present in altered T4*e*HS90 phage particles propagated in the restrictive host. Even though ten times more *amber* particles than wild-type particles were used in the experiment, very little activity was detected; less than 0.6% of the lytic activity of the wild type. A similar result was obtained with another *amber* mutant T4*e*H26.

When T4*e*HS90 phage is propagated in the permissive host, the infected cells lyse spontaneously. The lytic activities of lysate as well as of the isolated phage are poor (Fig. 1C). T4*e*HS90 *amber* mutant may be suppressed only partially in *E. coli* CR63, or the specific activity of the *amber* mutant lysozyme produced in the permissive host is low.

These results suggest that the lytic activity of isolated phage particles and that found free in the lysate may be directed by the same gene.

Thermostability of the lytic activities of lysates and of isolated phage

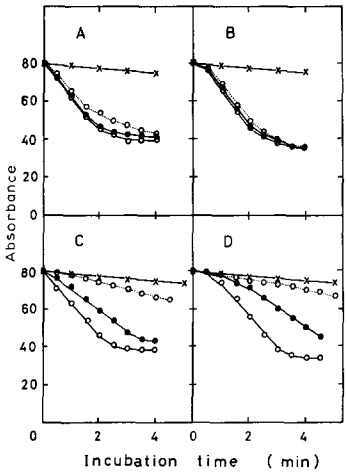
There is a possibility that the structural genes for free lysozyme and particle-bound lysozyme are different, but the function of the latter is affected by a mutation in the former. For instance, an *amber* mutation in the *e* gene might exhibit a polar effect on other genes, and as a consequence the lytic activity might not be detected in T4*e am* phage propagated in the restrictive host. In order to test this possibility, the thermostability of the lytic activities of lysates and of isolated phage was measured. If the lytic activities of lysates and of isolated phage are directed by two different structural genes, they may have rather different thermostabilities. As shown in Fig. 2, the lytic activities of lysates and of particles of the wild-type phage are relatively stable at temperatures of 45° or lower. The lytic activities of both lysates and of particles of T4*e*45 phage decreased with the preincubation at temperatures higher than 27°. Extents of inactivation of the lytic activities of lysates and of isolated phage are identical. It therefore seems that both the lytic activity found free in lysate and that of isolated phage are due to the same enzyme directed by *e* gene.

Binding of phage lysozyme to phage particles

Particle-bound lysozyme is not easily removed from phage particles by washing, and it becomes active only when phage particles are disrupted. This suggests that lysozyme is not merely bound to phage particles, but exists inside the particles. This notion is supported by the observation that free lysozyme does not bind irreversibly to mature phage particles.

In an experiment, the result of which is shown in Fig. 3, T4*e am* phage particles propagated in the restrictive host were incubated with phage-free lysate, and the binding of the free lysozyme to the phage particles was examined. In centrifuging at 30 000 × *g* for 60 min, about 17% of phage lysozyme sedimented with the phage particles. 75% of the lysozyme sedimented was removed by the first washing, and the remaining 25% was removed by the second washing. Phage particles subjected to 3 cycles of washing were free of lysozyme activity. From these results, it may be suggested that particle-bound lysozyme is the phage lysozyme which is incorporated into phage particles before the completion of phage maturation.

It was also demonstrated that egg white lysozyme is not irreversibly adsorbed to mature phage particles. After incubating T4*e am* phage with 5 μg/ml of egg white lysozyme in M9 medium, lysozyme-free particles were reisolated by 3 cycles of washing.



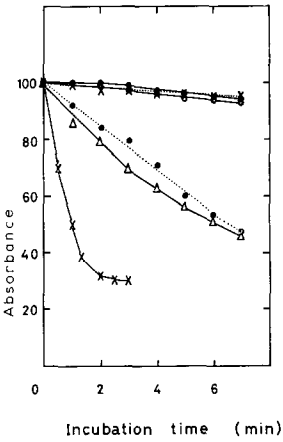


Fig. 2. Thermostability of the lytic activities of lysates and altered phage. T4e^+ and T4e45 phages were propagated in *E. coli* B at 27°. The isolated phages were altered in the presence of of pancreatic deoxyribonuclease (10 μg/ml) and 5 mM Mg^{2+} by freezing and thawing 5 times alternately in an acetone–dry-ice bath and in a 27° water bath. The assay of the lytic activity is described in METHODS. The test materials were incubated for 40 min at 27° (○—○), 37° (●—●) and 45° (○·····○) prior to addition to the assay mixture. The amount of test materials added to the assay mixtures (4 ml total vol.) were: $1 \cdot 10^{11}$ altered T4e^+; $2.5 \cdot 10^{11}$ altered T4e45; 0.1 ml of phage-free lysate (corresponding to approx. $5 \cdot 10^{10}$ phage); no addition (×—×). (A) Phage-free lysate of T4 wild type. (B) Altered T4 wild-type phage. (C) Phage-free lysate of T4e45. (D) Altered T4e45 phage.

Fig. 3. The absence of phage lysozyme irreversibly bound to phage particles *in vitro*. T4eHS90 was liberated from the restrictive host by the autolysis method. The particles ($5 \cdot 10^{10}$ per ml) were incubated in M9 medium with free lysozyme (phage-free lysate of wild-type T4: 1 ml of lysate corresponds to approx. $5 \cdot 10^{10}$ phage) at 37° for 90 min. The phage particles were washed 3 times by differential centrifugation, and altered by freezing and thawing as described in METHODS. The assay of lytic activity is described in METHODS. Test materials added to the assay mixture (4 ml total vol.) were: ×·····×, none; ○—○, $6 \cdot 10^{11}$ intact T4eHS90 (after incubation and washing; ●—●, $6 \cdot 10^{11}$ altered T4eHS90 (after incubation, washing and alteration). The following were also added to the assay mixtures (4 ml total vol.) as references: ●·····●, $8 \cdot 10^{10}$ altered T4e^+; △—△, 0.2 ml of phage-free lysate of wild-type T4 (corresponding to approx. $1 \cdot 10^{10}$ phage); ×—×, 10 μg of egg white lysozyme.

Thus, without complication of the results, the lysozyme treatment can be used to liberate T4e *am* phage from the restrictive host.

Some functional aspects of particle-bound lysozyme

It may be inferred that phage lysozyme is involved in 'lysis-from-without'. It was assumed that cell wall digestion by lysozyme of many virus particles leads to abrupt lysis (lysis-from-without)[3]. If the assumption is correct, T4e *am* phage deficient in lysozyme will not induce lysis-from-without. Fig. 4 shows that lysis-from-without is brought about by T4eHS90 phage, which is propagated in the restrictive host and is deficient in lysozyme. It is evident from this result that particle-bound lysozyme is not responsible for this phenomenon.

It was also assumed that the rigid innermost layer of the cell wall is pierced by the particle-bound lysozyme at the outset of infection[3]. However, it was demonstrated that T4e *am* phage particles liberated from the restrictive host by the autolysis method

are fully infectious, and these particles are devoid of lysozyme activity. These results may be taken to indicate that particle-bound lysozyme is not essential to phage infection.

It might be possible that phage can invade its host without digesting the rigid mucopolymer of the cell wall. The alternative explanation is that the rigid layer is digested at the time of infection by a phage enzyme different from lysozyme.

Presence of an enzymatic activity different from that of lysozyme in isolated T4 phage

It has been shown that particle-bound lysozyme is not essential to the invasion process of the host cell, and that the bound lysozyme does not account for lysis-from-

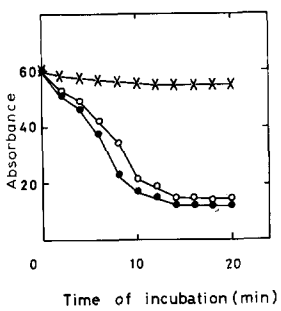


Fig. 4. Lysis-from-without by T4*e*HS90 phage propagated in the restrictive host. T4*e*HS90 was liberated from the restrictive host by the autolysis method. *E. coli* B cells in M9 medium supplemented with L-tryptophan (20 μg/ml) and 5 mM KCN were incubated with T4*e*HS90 at 37°, and the change of absorbance (at 660 mμ) of the mixture was followed. Multiplicity of infection: ×—×, 0; ○—○, 20; ●—●, 30.

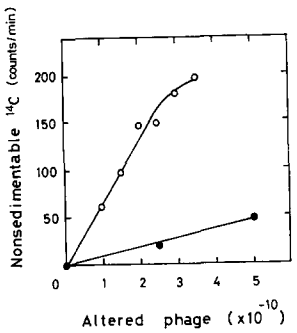
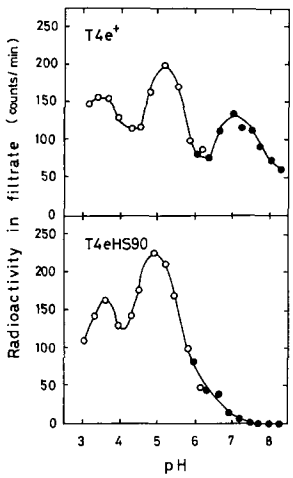


Fig. 5. Release of ^{14}C-labelled compounds from cell walls by varying amounts of altered phage. T4*e*HS90 propagated in the restrictive host was liberated by the lysozyme treatment. The preparation of altered phage and that of ^{14}C-labelled cell walls are described in METHODS. The incubation mixture (1 ml total vol.) contained 0.2 ml of ^{14}C-labelled cell wall preparation (4 000 counts/min), 0.01 M Tris-HCl buffer (pH 7), and altered phage. After incubating for 120 min at 37°, the mixture was centrifuged at 19 000 × *g* for 60 min at 4°. The supernatants were decanted, dried on aluminum planchets and counted. The cell wall concentration in one reaction mixture did not exceed $5 \cdot 10^9$ cell walls. ○—○, nonsedimentable ^{14}C produced by altered T4*e*$^+$ phage; ●—●, nonsedimentable ^{14}C by altered T4*e*HS90 phage propagated in the restrictive host.

without. In order to see whether these events proceed nonenzymatically or are due to another enzyme different from phage lysozyme, the degradation of ^{14}C-labelled cell walls by altered phages was examined by the method of BROWN AND KOZLOFF[2]. The cell walls were not digested by intact T4 phage in the absence of tryptophan, which is needed for phage adsorption.

As shown in Fig. 5, altered T4 wild-type phage digests the cell wall up to about 5%. The altered T4*e*HS90 phage lacking lysozyme also breaks down the cell wall to a lesser extent. This suggests the possible presence of an enzyme different from phage lysozyme. Fig. 6 shows the cell wall degradation by altered T4 phage as a function of pH of the assay mixture. There are 3 different pH optima for the cell wall degradation; pH 7 (Peak I), pH 5 (Peak II) and pH 3.5 (Peak III).

Peak I appears to represent phage lysozyme. This peak is absent in T4*e*HS90



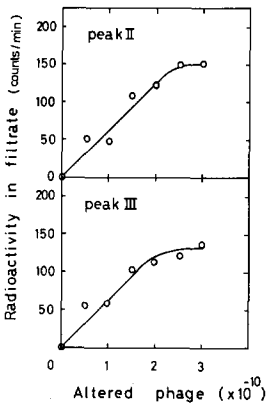


Fig. 6. The degradation of ^{14}C-labelled cell wall by altered phage as a function of pH of the assay mixture. T4e^+ and T4eHS90 were propagated in *E. coli* B. T4eHS90 was liberated from the host cell by the lysozyme treatment. The preparation of altered phage and the assay of cell wall degradation are described in METHODS. The incubation mixture (1 ml total vol.) contained 0.2 ml of ^{14}C-labelled cell wall preparation (4000 counts/min), 0.02 M buffer and $2 \cdot 10^{10}$ altered phage particles, and incubated for 60 min at 37°. Buffer used: ○—○, sodium acetate; ●—●, potassium phosphate.

Fig. 7. The degradation of ^{14}C-labelled cell wall by varying amounts of altered phage. T4eHS90 propagated in *E. coli* B was liberated by the lysozyme treatment. The preparation of altered phage and the assay of cell wall degradation are described in METHODS. The incubation mixture (1 ml total vol.) contained 0.2 ml of ^{14}C-labelled cell wall preparation (4000 counts/min), 0.02 M sodium acetate buffer and altered T4eHS90 propagated in the restrictive host, and was incubated for 60 min at 37°.

phage, which is propagated in the restrictive host and is devoid of particle-bound lysozyme.

Peak-II and -III activities are both proportional to the amount of altered phage (Fig. 7), and if the altered phage is heated to 100° for 5 min, these activities are completely destroyed. The reactions appear to be enzymatic, but it is not known whether the Peak-II and -III activities are due to one enzyme or to two. In contrast to phage lysozyme, the enzyme lyses neither chloroform-treated cells of *E. coli* nor intact cells of *M. lysodeikticus*. No comparable enzyme could be detected in uninfected cell extract.

DISCUSSION

Since the discovery of an enzymatic activity in isolated T-even phages[1,2], it has been assumed that this enzyme may play some role in the invasion process of the host cell. The enzyme was characterized as a lysozyme[4]. It has been assumed that particle-bound lysozyme digests part of the rigid layer of the cell wall, and the phage invades its host through the hole. If the fraction of the digested rigid layer is so great that the wall can no longer withstand the osmotic forces, the cell lyses almost immediately without viral production (lysis-from-without).

However, the evidence presented in this paper does not seem to support this

hypothesis. Lysozyme activity is absent in T4*e am* phage particles propagated in the restrictive host, and the thermostability of particle-bound lysozyme is almost identical with that of phage lysozyme found free in lysate. The phage particles lacking lysozyme are fully infectious, and they induce lysis-from-without. These results may be taken to indicate that particle-bound lysozyme is not essential to the invasion process of the host cell, nor does it account for lysis-from-without. It follows from these results that phage might be able to invade its host without digesting the rigid mucopolymer of the cell wall. Another possible explanation of the results is that the rigid layer might be digested at the time of infection by a phage enzyme different from lysozyme.

To test the possibilities, the degradation of ^{14}C-labelled cell walls by altered phage was examined. An enzymatic activity different from that of lysozyme was found. The enzymatic activity attacks *E. coli* cell walls at pH 5 and at pH 3.5, and it can be found in the phage particles lacking lysozyme. In contrast to lysozyme, the enzyme lyses neither chloroform-treated cells of *E. coli* nor intact cells of *M. lysodeikticus*.

Some attempts were made to elucidate the function of the enzyme. On the assumption that lysis-from-without is due to the enzyme, mutants unable to cause lysis-from-without were selected. Some phage mutants were obtained, which do not exhibit this phenomenon in the crude lysate; but when isolated from the lysate, they induce the normal extent of lysis-from-without. Thus, they are not truly deficient in this respect, and moreover, they carry the enzymatic activity.

An attempt was also made to examine the enzymatic activity in T4*am*N93 (gene 11) and T4*am*N69 (gene 12) phages propagated in the restrictive host. Gene 11- and 12-defective phages have no host-killing activity[12], and they do not cause lysis-from-without (unpublished result). The enzyme could be detected in both defective T4*am*N93 and T4*am*N69 phages. However, the possibility must be taken into account that gene 11 and 12 products are not the direct causative agents for these defective functions, but that they may be involved in intermediate steps in the attachment process[12].

The mode of action or the function of the enzyme remains to be elucidated.

After submitting the manuscript of this paper, the author noticed that similar observations had been published[13].

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