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LYSOZYME ACTIVITY OF BACTERIOPHAGE T4 GHOSTS

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Bacteriophage T4 ghosts were found to possess lysozyme (mucopeptide *N*-acetylmuramoylhydrolase, EC 3.2.1.17) activity. This enzyme is probably responsible for the lysis from without, observed at high multiplicity of infection, a process independent of the presence of the *e* gene product which is also a lysozyme. The ghost lysozyme and *e* lysozyme differed with respect to their requirements for maximal catalytic activity and to some extent in substrate specificity. The ghost lysozyme was released from phage particle by the action of Triton X-100.

Introduction

Bacteriophage T4 can lyse bacterial cells by two different processes [1]: lysis from within and lysis from without. Lysis from within occurs at the end of the latent period and it has been shown previously [2] that the lysozyme synthesized under the control of the *e* gene of bacteriophage T4 is responsible for this process. Lysis from without is the decomposition of bacterial cells at high multiplicity of infection and it is independent of the presence of the *e* gene product [3]. It was shown [4] that wild-type phage T4 and its *e* mutants release similar cell wall materials in the course of phage adsorption, indicating the presence of phage-associated lytic activity. Recently, Kao and McClain [5] suggested that this activity may be attibuted to the product of gene *5* which is an integral component of the virion baseplate, though they have not determined what the actual substrate of this enzyme is.

In this paper we show that the lytic activity associated with bacteriophage T4 particle is a lysozyme (mucopeptide *N*-acetylmuramoylhydrolase EC 3.2.1.17) and we compare its properties with the well-characterized properties of the *e* gene product [6].

Materials and Methods

Bacteriophage. Bacteriophage T4 amber mutant in gene *e* (T4am H26) was obtained from G. Fletcher. The temperature-sensitive mutant in gene *5*-5ts1 and mutants which in addition to that mutation are defective in the production of the *e* gene product (5ts1 eL1a-*e* amber mutant and 5ts1 eG19-*e* deletion mutant) were from W. McClain. 5ts1 mutants form normal plaques at 30°C and very minute plaques at 42°C. Wild-type T4D was from our collection.

Bacteria. Escherichia coli CR63, a permissive host for amber mutants, and *E. coli* B^E, a non-permissive host for amber mutants were used for phage growth and plating. The strains were obtained from E. Kellenberger.

Media. Buffer - 7 g Na_2HPO_4 2 H_2O/3 g KH_2PO_4/

Abbreviations: GlcNAc, *N*-acetyl-D-glucosamine; MurNAc, *N*-acetylmuramic acid; anMurNAc, 1,6-anhydro-*N*-acetylmuramic acid; muropeptide C6, GlcNAc-MurNAc-L-Ala-D-Glu-meso-diaminopimelic acid-D-Ala; muropeptide C3, dimer of C6 units connected by D-D peptide bonds between D-Ala and meso-diaminopimelic acid; muropeptide CA, GlcNAc-anMurNAc-L-Ala-D-Glu-meso-diaminopimelic acid-D-Ala; muropeptide CB, GlcNAc-MurNAc-GlcNAc-anMurNAc in which carboxyl groups of MurNAc and anMurNAc are substituted by L-Ala-D-Glu-meso-diaminopimelic acid-D-Ala.

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4 g NaCl/10^{-3} M $MgSO_4$/1 l H_2O. Broth - M9A broth, which is an M9 broth supplemented with 5 ml of a 20% solution of Difco casamino acids per 100 ml M9 used. Plating - top agar: the same as broth, but in addition contains 7 g/l Difco agar; bottom agar: the same as broth, but in addition, contains 11 g/l Difco agar. For plating of gene *e* deletion mutants citrate top agar supplemented with egg-white lysozyme was used [7].

Buffers for activity measurements. The buffers of constant ionic strength were prepared according to Miller and Golder [8]: sodium acetate/acetic acid/NaCl (pH 4.0–5.5), mono- and disodium phosphate/NaCl (pH 6.0–7.5), HCl/veronal/NaCl (pH 8.0–9.0), glycine/NaOH/NaCl (pH 9.0).

Preparation of bacteriophage T4 phenotypic products. Wild-type T4D and *e* amber mutant: Cultures of *E. coli* B^E grown at 37°C to $2 \cdot 10^8$ bacteria/ml in M9A broth were infected with a phage at a multiplicity of infection of 5. 8 min after the first infection, the culture was superinfected at the same multiplicity of infection (lysis inhibition) and incubated for about 2 h. The infected bacteria were centrifuged off at room temperature and resuspended in phosphate buffer. A few drops of chloroform and DNAase (20–50 μg/ml) were added and the mixture was left overnight. Finally, the bacterial debris was pelleted.

5ts1, 5ts1 eL1a and 5ts eG19 mutants: the 5ts form of the product of gene *5* results in a partial defect in lysis inhibition [9], therefore, another procedure for the preparation of phage lysates was employed. Cultures of *E. coli* B^E grown at 30 or 42°C to $4 \cdot 10^9$ cells/ml in M9A broth were infected with a phage at a multiplicity of infection of 1 and incubated for 90 min with very gentle shaking. Further procedure was as above.

All phage mutants were purified by CsCl gradient sedimentation.

Preparation of phage ghosts. 10 g saccharose were added to 5 ml phage suspension ($2 \cdot 10^{13}$ plaque forming units/ml) in 0.05 M Tris-HCl buffer/0.01 M EDTA and the mixture was agitated at room temperature until the saccharose was completely dissolved. 5 ml glycerine were added and the viscous mixture was added dropwise to 200 ml chilled, distilled water agitated by means of a magnetic stirrer. 1 ml 1 M $MgSO_4$ and 0.1 ml DNAase (10 mg/ml) were added and the stirring was continued at 4°C for 1 h. The ghosts were concentrated by centrifugation at 53 000 × *g* at 10°C for 90 min and separated from the intact phages by CsCl gradient sedimentation (140 000 × *g* at 4°C for 3 h) in the gradient range 0–1.5 g/c.c. In these conditions the intact phages sediment at the bottom of the tube, while the ghost band is situated in the central part. The purified phage ghosts contained about one intact phage per 10^4 ghost particles.

Determination of ghost titer. The titer of bacteriophage ghosts was assessed by means of protein determination according to Heil and Zillig [10]. 10 μl of the ghost mixture was mixed with 10 μl of Laemmli lysing buffer [11] in an Eppendorrf tube and the tightly sealed tube was heated for 5 min at 100°C. The sample was then applied to nitrocellulose foil and stained with Amido Black. The stained portion of the foil was extracted with 1.5 ml absolute formic acid and the absorbancy at 630 nm was measured. The standard curve was prepared for the fresh phage stock (1 mg T4 protein corresponds to $1.5 \cdot 10^{11}$ plaque forming units).

Enzymes. Phage T4 lysozyme (*e* gene product) was obtained by a method used previously [12]. λ endolysin was purified as described by Taylor [13]. Hen egg-white lysozyme and DNAase were from Sigma Chemical CO. (St. Louis, MO, U.S.A.).

Enzyme substrates. Soluble chitin fragments containing five to eight *N*-acetylglucosamine units were obtained according to Sharon et al. [14]. *Bacillus subtilis, Micrococcus lysodeicticus* and *Escherichia coli* mureins were prepared by methods used previously [13,15].

Materials. Triton X-100 was from Polysciences, Inc. (Warrington, U.S.A.). Tris-HCl. Amido Black and sucrose were from Serva Feinbiochemica (Heidelberg, F.R.G.). All inorganic chemicals were from POCH (Gliwice, Poland). Reference muropeptides C3, C6 and CA were obtained as previously described [13, 16].

Analytical methods. For the quantitative determination of bacteriolytic activity, [^{3}H]meso-diaminopimelic acid-labelled murein-lipoprotein was used and the test was carried out according to Höltje et al. [17]. The standard curve for hen egg-white lysozyme was prepared. One bacteriolytic unit corresponded to the bacteriolytic activity of 1 μg of the lysozyme. *N*-Acetylamino sugars were determined according to

Ghuysen et al. [18]. Thin-layer chromatograms of muropeptides were done on cellulose (5578, Merck) in *n*-butan-1-ol/acetic acid/water (4 : 1 : 5, upper phase) solvent system and developed twice at 30°C. The muropeptides were detected by 0.2% ninhydrin in acetone.

Results

Specificity requirements of the lytic enzyme of bacteriophage T4 ghosts. The incubation of bacteriophage T4 ghosts (wild type or *e* mutant) with *E. coli* B murein leads to the formation of low-molecular weight fragments, mainly C3 and C6 muropeptides (Fig. 1), that is, the same muropeptides which are produced upon the incubation of *E. coli* B murein with hen egg-white lysozyme or bacteriophage T4 lysozyme (*e* gene product, later in the text called *e* lysozyme). *e* lysozyme is much more specific as regards the substrate than hen egg-white lysozyme, as it catalyses the hydrolysis of only those murein chains in which *N*-acetylmuramic acid is substituted by peptide side chains L-Ala-D-Glu-meso-diaminopimelic acid-D-Ala [19]. Hence, *M. lysodeicticus* murein, having a different composition of peptide chains, is a very bad substrate for *e* lysozyme and low molecular-weight murein fragments are not produced upon the incubation of this murein with *e* lysozyme. On the other hand, hen egg-white lysozyme hydrolyses *M. lysodeicticus* murein to low molecular-weight fragments and it also hydrolyses chitin fragments. The results obtained for bacteriophage T4 ghosts (Table I) indicate that their lysozyme is even more specific than *e* lysozyme, as it does not catalyze the hydrolysis of *B. subtilis* murein whose structure is essentially the same as *E. coli* murein and which is a substrate for *e* lysozyme.

To check whether only the intact murein is the substrate for the lysozyme of phage T4 ghosts, we have also used the low molecular-weight substrate of

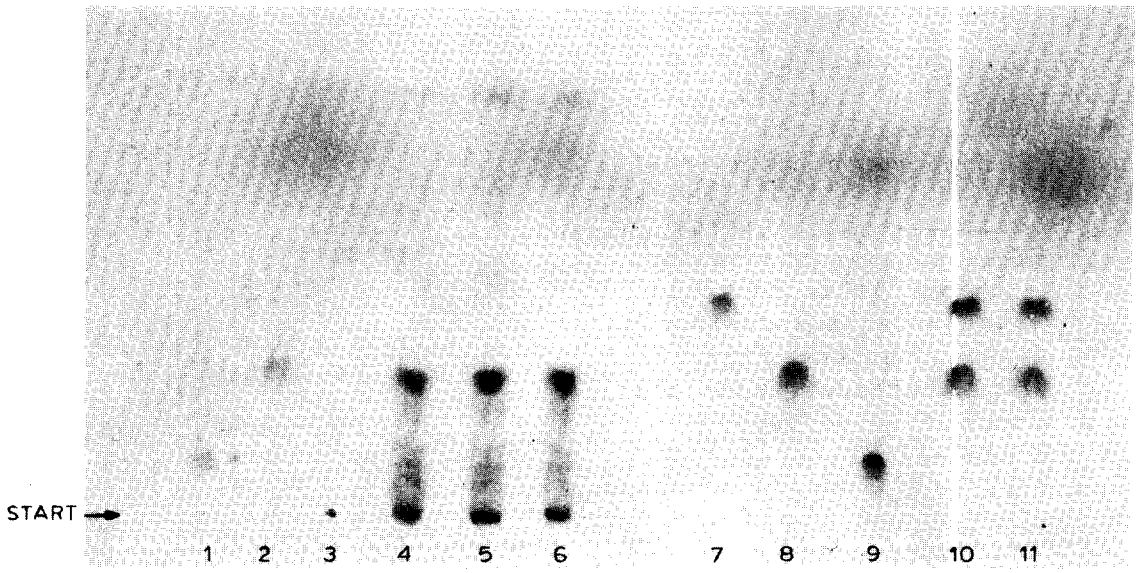

Fig. 1. Thin layer chromatogram representing the digestion of *E. coli* B murein and muropeptide CB by phage T4 ghosts. Murein (100 μg) and muropeptide CB (20 μg) were digested by phage T4 ghosts (about 10^{10} ghosts corresponding to 5 bacteriolytic units) in 30 μl of 50 mM ammonium acetate, pH 5.5/0.2% Triton X-100 at 37°C for 12 h. Ammonium acetate was removed in vacuum. For comparison, murein and muropeptide CB were digested with 5 bacteriolytic units of *e* lysozyme. 1. muropeptide C3 (10 μg); 2. muropeptide C6 (5 μg); 3. native murein (100 μg) - no colour reaction products with ninhydrin; 4. the products of digestion of murein by *e* lysozyme; 5. the products of digestion of murein by the ghosts of bacteriophage T4 amber *e* mutant; 6. the products of digestion of murein by the ghosts of 5ts1 eG19 bacteriophage T4 mutant grown at 30°C; 7. muropeptide CA (10 μg); 8. muropeptide C6 (5 μg); 9. muropeptide CB (5 μg); 10. the products of digestion of CB by the ghosts of bacteriophage T4 amber *e* mutant; 11. the products of digestion of CB by *e* lysozyme.

TABLE I

DIGESTION OF MUREINS, MUROPEPTIDE CB AND CHITIN OLIGOSACCHARIDES BY VARIOUS LYSOZYMES

+, digested (in case of mureins to low molecular weight fragments, detected crhomatographically). –, not digested. Reaction conditions: 100 μg murein or 20 μg muropeptide CB were digested by 10 bacteriolytic units of lysozymes and analysed chromatographically as described under Fig. 1. 10 μl (50 μg) chitin fragments were incubated with 10 bacteriolytic units of lysozymes in 0.1 M ammonium acetate buffer, pH 5.5 and 7.0 for 12 h. The increase of free *N*-acetylglucosamine groups was determined colorimetrically [18]. All the reaction mixtures contained 0.2% Triton X-200.

Lysozyme source	Substrate types				
	E. coli murein	Muropeptide CB	*B. subtilis* murein	*M. lysodeic ticus* murein	Chitin fragments
Bacteriophage T4 ghosts	+	+	–	–	–
T4 phage *e* gene product	+	+	+	–	–
Hen egg-white	+	+	+	+	+

e lysozyme [20]. This is muropeptide CB obtained upon the digestion of *E. coli* B murein with phage λ endolysin and it contains only one bond sensitive to the *e* lysozyme. Muropeptide CB is digested in the same way by both lytic enzymes of bacteriophage T4 (Fig. 1).

Effect of Triton X-100. The addition of Triton X-100 to the mixture of murein and phage ghosts leads to over 10-fold increase of the murein hydrolytic activity (Fig. 2). By contrast, the activity increase of *e* lysozyme in the presence of Triton X-100 does not exceed 50%. We think that this drastic increase of activity of the phage ghost lysozyme may be an indirect effect due to the solubilization of the protein responsible for the lytic activity. Our assumption is supported by the observation that the phage treated with Triton X-100 and subsequently centrifuged down, contains less than 10% of the original activity, while most of the activity is found in the supernatant.

Activity as a function of pH and ionic strength. There was a significant difference in the pH optimum of murein hydrolytic activity for *e* lysozyme and bacteriophage ghosts when this value was determined at constant ionic strength (Fig. 3). Under our conditions, *e* lysozyme exhibits the highest activity at pH 7.6 (at $I = 0.1$) while bacteriophage ghosts have the highest activity in the pH range 5.5–6.0 (at the same ionic strength value). At pH 7.6 the activity of bacteriophage ghosts is about 50% of its maximum value. The pH range in which bacteriophage ghosts catalyse the hydrolysis of murein is fairly wide, from 2.5–8.5

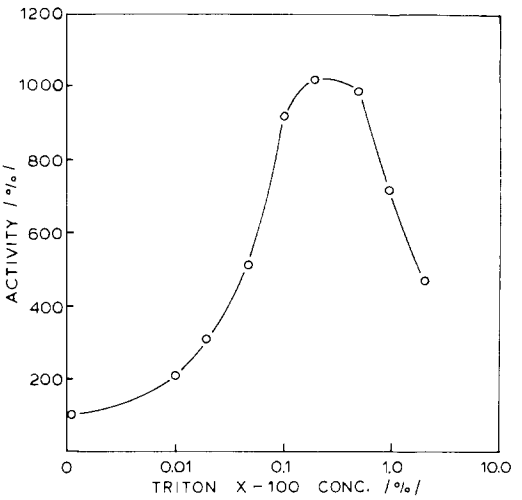

Fig. 2. The effect of Triton X-100 on the murein hydrolytic activity of bacteriophage T4 ghosts. [^{3}H]meso-diaminopimelic acid-labelled murein-lipoprotein sacculi (10 μl equivalent to about 10^4 cpm) were incubated with bacteriophage ghosts (3 bacteriolytic units corresponding to the release of about 80% radioactivity to the solution in the optimum conditions) in a Miller and Golder buffer, pH 6.0, ionic strength - 0.1 containing varying concentrations of Triton X-100 (the total volume - 100 μl) for 20 min at 37°C. The reaction was stopped by the addition of 10 μl of 50% TCA. 10 μl bovine serum albumin (20 μg/μl) were added and the precipitate was centrifuged off. 75 μl of the supernatant were mixed with 750 μl of 0.1 N NaOH and the radioactivity was measured in a liquid scintillation counter. Activities are expressed as a percentage of the activity without Triton X-100 in the reaction mixture.

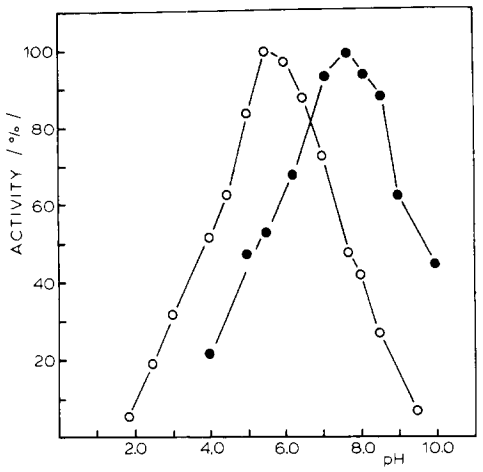

Fig. 3. pH activity curves of bacteriophage T4 ghost lysozyme and *e* lysozyme. The experimental details are given below Fig. 2. Miller and Golder buffers of ionic strength - 0.1 were used. ○———○, the ghosts of bacteriophage T4 amber *e* mutant; ●———●, *e* lysozyme.

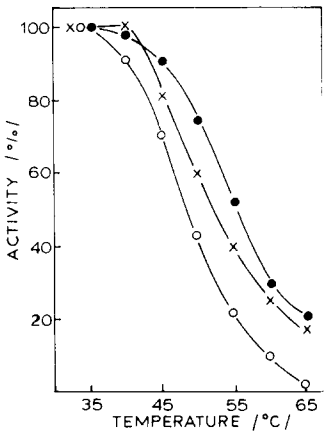

Fig. 4. Thermal stability of the lytic activity of bacteriophage T4 ghosts. Bacteriophage ghosts or *e* lysozyme (about 3 bacteriolytic units) were incubated for 10 min in a Miller and Golder buffer, pH 6.0, ionic strength - 0.1, at each temperature indicated. After the incubation the solution was cooled in an ice-water bath and the residual activity was measured as described below Fig. 2. Activities are expressed as a percentage of the activity at 35°C. ●———●, the ghosts of bacteriophage T4 amber e mutant; X———X, the ghosts of 5ts1 eL1 mutant grown at 42°C; ○———○, e lysozyme.

(about 20% of maximum activity at these extreme pH values).

The optimum ionic strength for bacteriophage ghosts lysozyme is in the range 0.05–0.15 for pH values 6.0–8.0. The activity decreases to about 50% of the maximum value at $I = 0.015$ and $I = 0.25$, and the enzyme is practically inactive below $I = 0.005$ and above $I = 0.5$. The relation between ionic strength and *e* lysozyme activity is similar, though in this case the optimum ionic strength is shifted a little towards lower values - $I = 0.025$–0.10.

Effect of Mg^{2+}. Jensen and Kleppe [6] found that magnesium increases the activity of *e* lysozyme towards chloroform-treated *E. coli* B cells or towards *E. coli* B murein. Bacteriophage ghosts activity does not change when this ion is added to the reaction mixture in concentrations described by these authors as activating (0.5–5.0 μM at $I = 0.1$, pH 6.0–8.0).

Thermal stability of the lysozyme activity. The lysozyme of bacteriophage ghosts is more resistant to the effect of elevated temperature than *e* lysozyme (Fig. 4). The inactivation starts above 45°C, but at 70°C the residual activity is still about 20% of the maximum value.

Activity 5ts1 mutants. Kao and McClain [5] described the isolation and characterization of a new class of phage T4 mutants that permit lysis of infected cell in the absence of *e* lysozyme. Their results indicate that the product of gene *5* may be a candidate for the second lytic enzyme of bacteriophage T4. 5ts1 mutants form normal plaques at the permissive temperature of 30°C, and tiny plaques at 42°C. We have checked whether this behaviour of 5ts1 phages may be correlated to the inactivation of the lytic enzyme of bacteriophage ghosts. It was found that in CsCl gradient, 5ts1 phages behave in a different way to wild-type T4 or its *e* amber mutant. Instead of one band of the purified phage, three bands with positions corresponding to phage, ghosts and free proteins (moving from bottom to top of the tube) were observed. This was observed irrespective of the temperature at which the mutant was grown and irrespective of the mutant type (5ts1, 5ts1 eL1 or 5ts1 eG19), though the ratio of the ghost band width to the phage bandwidth was much higher for the mutants grown at 42°C. The lytic activity was found in each of the three fractions for mutants grown at 30°C, as well as at 42°C. The relation between the lytic activity and temperature for 5ts1 phages and

phage ghosts is almost the same as for amber *e* phage ghosts (Fig. 4), suggesting that the thermosensitivity is not a direct effect of thermal instability of the lytic enzyme.

Discussion

The results reported here confirm the earlier observations that the lytic activity is associated with bacteriophage T4 particles. The bonds joining the lytic protein to the phage particle are relatively strong, as they withstand high salt concentration (CsCl gradient sedimentation) and fairly harsh treatment during the preparation of phage ghosts. The effect of Triton X-100 triggering the release of the enzyme, leads to speculations on the possible role of the lipophilic components of bacterial envelopes aiding the enzyme to attain its target, murein, upon the attachment of the phage to a bacterial cell. Whether Triton X-100 only releases the protein from phage particles or releases and activates it, is not yet known and we have to wait for the answer until the pure protein is obtained.

The lytic activity of phage particles is a lysozyme which is even more specific as regards the substrate than *e* lysozyme. It does not hydrolyse *Bacillus subtilis* murein to low-molecular weight fragments, as is observed for *e* lysozyme. The particle-associated lysozyme differs notably from *e* lysozyme in some physicochemical properties such as pH optimum, temperature dependance and effect of magnesium on the activity. These values may not be exactly the same for the isolated lysozyme protein because the incorporation into phage particles may alter some of its properties and we cannot exclude the possibility that Triton X-100 releases a subunit of a phage particle (SDS-polyacrylamide gel electrophoresis of Triton X-100 extract reveals the presence of at least ten protein bands - data not shown). However, by way of analogy, the binding of *e* lysozyme to a solid support [21] only slightly changes its properties.

The behaviour of 5ts1 mutants in CsCl gradient suggests that the heat sensitivity of these phages is not a direct effect of the protein inactivation, but rather of the defective assembly. This is in agreement with the results of Kao and McClain [5] showing that the particle-associated lytic enzyme (the product of gene *5* according to suggestions of the authors) can replace *e* lysozyme in 5ts1 mutants, probably because it is in a soluble form. We think that the confirmation of the role of gene *5* product as the lytic enzyme requires its biochemical purification, as the observed phenotypic effects for 5ts1 mutants may be caused indirectly and we pursue this subject. In our opinion, the purification may be facilitated by the following facts: (i) Triton X-100 extract is relatively stable as regards the lytic activity (no loss of activity during a monthly storage at 4°C). (ii) treatment with strong dissociating agents (6 M urea, formamide) does not inactivate the lytic protein.

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