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Purification and Properties of Phage P22-induced Lysozyme*

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G. R. KOTESWARA RAO AND D. P. BURMA

From the Department of Biochemistry and Biophysics, Institute of Medical Sciences, Banaras Hindu University, Varanasi-5, U.P., India

SUMMARY

Phage P22 induces a lysozyme in *Salmonella typhimurium* cells toward the later stage of its multiplication. P22 lysozyme has been purified about 1000-fold starting from the lysate of C_1 (clear plaque-forming mutant of phage P22)-infected cells. The enzyme has an optimum pH between 7 and 8 and its activity is dependent on the ionic strength of the assay medium. Salts like $MgCl_2$, NaCl, and KCl are inhibitory to the lysozyme. Gram-negative cells act as better substrates for the lysozyme than do gram-positive cells The enzyme has a molecular weight of about 2×10^4 and rapidly looses its activity at temperatures higher than 40°. The properties of P22 lysozyme have been compared with those of λ and T4 lysozymes. All three lysozymes have more or less the same molecular weight and have similar properties although P22 and T4 lysozymes seem to be closer than P22 and λ lysozymes.

Temperate phage P22 of *Salmonella typhimurium* (strain LT2) has more or less the same molecular dimensions as those of temperate phage λ of *Escherichia coli* and behaves like λ in many respects although there are differences in detail. Unlike λ DNA, P22 DNA has no sticky ends (1). In some respects P22 is closer to virulent T-even phages of *E. coli* than to λ, *e.g.* DNA of P22 is circularly permuted like the DNA of T-even phages (1). Therefore it was of interest to know the properties of phage P22-induced lysozyme and to compare those with the properties of λ and T-even phage-specific lysozymes.

For the lysis of the host it is obligatory for the phage to produce a lysozyme toward the end of its multiplication. Lysozyme is not normally detectable in the host and is induced by the phage genome. Lysozyme of phage T2 has been partially purified and its properties have been described (2). T7 lysozyme has also been partially purified (3). Both T4 lysozyme (4) and λ lysozyme (5-7) have been purified to homogeneous state and their properties have been investigated in detail. In the present paper the method of purification of P22 lysozyme to a highly purified state will be described and its properties will be compared with those of T4 and λ lysozymes. It should be mentioned in this connection that Cohen (8) while studying delayed lysis with a mutant of phage P22, measured the lysozyme activity induced in LT2 following infection with lys^+ phage (wild type which lyses the host) and a lys^- mutant (which fails to lyse the host).

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MATERIALS AND METHODS

S. typhimurium (strain LT2) and the clear plaque-forming C_1 mutant of phage P22 were kindly supplied by Professor M. Levine of the University of Michigan, Ann Arbor, Michigan. *E. coli* B and *Micrococcus lysodeikticus* were obtained from Dr. M. F. Singer of the National Institutes of Health, Bethesda, Maryland.

Crystalline preparations of egg white lysozyme, trypsin, cytochrome *c*, pancreatic ribonuclease were obtained from Sigma. Crystalline serum albumin and ovalbumin were the products of Nutritional Biochemicals and Biochemicals Unit, India, Council of Scientific and Industrial Research, respectively. Penicillin G was a commercial preparation of Sarabhai Chemicals, India. Sephadex G-25 and G-100 were supplied by Pharmacia. Amberlite IRC-50 was a product of British Drug Houses, England.

Growth of Cells—*S. typhimurium* (LT2) cells were grown at 37° with aeration in a synthetic medium containing K_2HPO_4, 10.5 g; KH_2PO_4, 4.5 g; sodium citrate-$5H_2O$, 0.47 g; $MgSO_4$-$7H_2O$, 0.1 g; and $(NH_4)_2SO_4$, 1 g in 1 liter of water. Glucose was added at a concentration of 0.2%.

Preparation of Cell-free Extracts—Extracts of noninfected and phage-infected cells were made by grinding for 10 min with alumina A-305 of Alcoa (2 g per g of cells, wet weight) in a mortar with pestle at 0°. Both noninfected and phage-infected cells, collected from 400-ml samples (Fig. 1), were ground with alumina and then extracted with 3 ml of 0.05 M Tris, pH 7.4, containing 2×10^{-4} M mercaptoethanol. Cell debris were removed by centrifugation at 20,000 × *g* for 20 min and the supernatant fluid was used as extract.

Preparation of Substrates for Lysozyme—Exponentially growing cells of LT2 (5×10^8 per ml) were harvested by centrifugation at 0° for 15 min at 5000 × *g* and suspended in half the volume of chloroform-saturated 0.01 M Tris, pH 8 (10^9 cells per ml). The suspension was shaken occasionally during 30 to 40 min at 25°. The chloroform-treated cells were washed twice with 0.01 M Tris, pH 8. These cells could be stored at −20° for several days. Before assay of the enzyme the frozen cells were thawed and suspended in 0.025 M Tris, pH 8.

The procedure for growing *E. coli* B and the method of treatment of the cells with chloroform were the same as described for LT2.

M. Lysodeikticus cells were grown in a medium containing

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tryptone, 5 g; yeast extract, 5 g; dextrose, 1 g; and K_2HPO_4, 1 g in 1 liter of water. Exponentially growing cells of *M. lysodeikticus* were harvested by centrifugation at 5000 × *g* for 15 min, washed twice with 0.01 M Tris, pH 8, and suspended in 0.025 M Tris, pH 8. The suspension was directly used as the substrate without any chloroform treatment.

Assay of Lysozyme—The activity of lysozyme was determined by a modification of the turbidometric method used by Sekiguchi and Cohen (9). Chloroform-treated cells were incubated at 25° with an appropriate amount of extract in 0.025 M Tris, pH 8 (3 ml total volume), and the decrease in turbidity was measured at 470 nm at 30-sec intervals for 5 min. The concentration of the chloroform-treated cells (*E. coli* or *S. typhimurium*) or non-treated *M. lysodeikticus* cells in the reaction mixture was adjusted to an absorbance of 0.6. The rate of reaction calculated from the change in absorbance in the linear region of the rate *versus* time curve has been used to express the activity of the enzyme. One unit of enzyme is defined as that which produces a change in absorbance of 1 per min under the above conditions.

RESULTS

Time Course of Induction of Lysozyme

Before starting to purify the phage P22-specific lysozyme the time course of induction of the enzyme was studied. The clear plaque-forming C_1 mutant of phage P22 was used for infection. The amount of lysozyme detectable in the cells as well as that released into the growth medium were simultaneously measured. The experimental details have already been described under "Materials and Methods." The results are presented in Fig. 1. There is no detectable amount of lysozyme present in the noninfected host (not shown) and detectable amount of lysozyme is formed only after 15 min following infection. During the next 15 min there is continued synthesis of lysozyme but beyond this period the amount of lysozyme measurable in the cells becomes less, perhaps as the result of the partial lysis of the cells. This is substantiated by the fact that increasing amount of lysozyme is detectable in the medium even up to 50 min. The total amount of enzyme present in the medium at 50 min is 7 times as much as that present in the intact cells at 30 min following infection. These results clearly pointed out that the phage-infected cells would be a rather poor source for the purification of the enzyme. Actually the medium after complete lysis of the host was used as the starting material for the purification of the lysozyme.

Purification of Lysozyme

Lysozyme was purified from the lysate of C_1-infected cells of LT2. The procedure is described below. All the operations were carried out at 0° unless otherwise stated.

Preparation of Lysate—LT2 cells were grown in synthetic medium (4 liters) up to 6.5 × 10^8 cells per ml under aeration at 37° and infected with C_1 at a multiplicity of infection of 10. After allowing 3 min for adsorption, aeration was continued until lysis of the cells took place, *i.e.* at about 60 to 70 min following infection. To this lysate about 5 ml of chloroform were added and the mixture was shaken vigorously to lyse the unlysed cells. The lysate could be stored at 4° for several days without any loss of activity. The cell debri was allowed to settle to the bottom and the clear supernatant fluid was collected by decantation and then centrifuged at 5000 × *g* for 20 min. The lysate

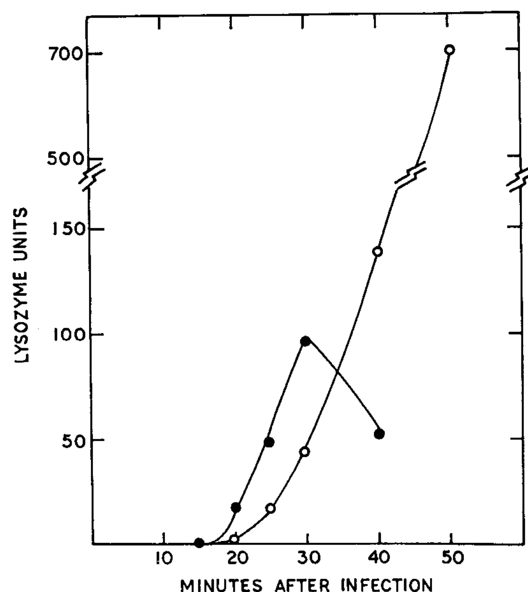

FIG. 1. Induction of lysozyme in P22-infected cells of LT2. Exponentially growing cells (6.5 × 10^8 per ml) were infected with C_1 at multiplicity of infection of 10. Samples (400 ml) were collected at requisite times and chilled immediately to 0°. The cells were collected by centrifugation for 15 min at 5000 × *g*, the supernatant fluid was stored at 0° for assay. The extract of cells was prepared as described under "Materials and Methods." The supernatant fluid as well as the cell extracts were used for the assay of lysozyme. ●, intracellular activity; ○, activity in medium.

containing 1½ to 2 units of lysozyme per ml was subjected to ion exchange chromatography.

Amberlite IRC-50 Column I—Amberlite IRC-50 (100 to 200 mesh) was washed according to the procedure of Hirs (10) and packed into a column (2.5 × 23 cm) which was equilibrated with 0.1 M sodium phosphate, pH 5.8. The lysate (4 liters) was diluted with an equal volume of cold distilled water and the pH of the lysate was adjusted to 5.8 with 10% glacial acetic acid. The lysate was passed through the column at a flow rate of 400 to 500 ml per hour. Then the column was washed with 4 to 5 column volumes of 0.1 M sodium phosphate, pH 6.5, containing 0.001 M $MgSO_4$. The bulk of the protein was removed by washing (Fig. 2). The adsorbed enzyme was eluted with the same buffer containing 0.5 M NaCl. Fractions (15 ml) were collected and the enzyme could be recovered in a total of 6 to 7 fractions (total volume, about 100 ml). The recovery of the enzyme was about 85 to 90% and it was purified about 90-fold by this single step.

Sephadex G-25 Column—Sephadex G-25 was swollen in water for 2 days and the fine particles were removed by decantation. A column (2.5 × 48 cm) of Sephadex G-25 was packed and equilibrated with 0.02 M sodium phosphate, pH 6.5, containing 0.001 M $MgSO_4$. The IRC-50 I eluate (100 ml) was passed through this column to lower the salt concentration. The column was eluted with the equilibrating buffer. The active fractions were pooled. The salt concentration in the pooled fraction was 0.15 to 0.2 M. There was some loss of enzyme activity by this procedure though a slight purification was achieved.

Amberlite IRC-50 Column II—The eluate (100 ml) from Sephadex G-25 was diluted with equal volume of water and the pH was adjusted to 5 with glacial acetic acid. The eluate was again

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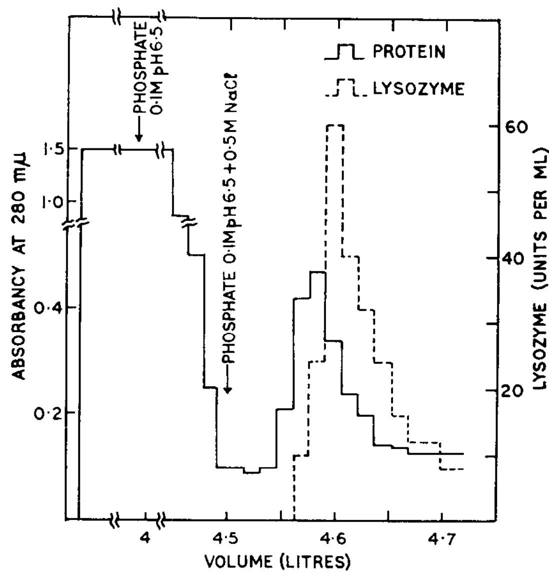

FIG. 2. Purification of P22 lysozyme on Amberlite IRC-50 I column. Lysate (4 liters) of C_1-infected LT2 cells was diluted with an equal volume of water and its pH adjusted to 5.8 before passing through the IRC-50 (100 to 200 mesh) column (2.5 × 23 cm). The detailed procedure has been described in the text.

TABLE I

Purification of P22 lysozyme

Specific activity has been expressed as units of enzyme per absorbance of unity at 280 nm as measured in Zeiss ultraviolet spectrophotometer.

Fraction	Total	$A_{280}:A_{260}$	Specific activity
	units		
Lysate	6000	0.51	1
Amberlite IRC-50 I	5300	0.70	90
Sephadex G-25	4700	0.83	120
Amberlite IRC-50 II	4100	1.20	970

passed through a IRC-50 column (1.5 × 20 cm) which was equilibrated with 0.1 M sodium phosphate, pH 5.8. The column was washed with 5 column volumes of 0.1 M sodium phosphate, pH 6.5, containing 0.001 M $MgSO_4$ and eluted with the same buffer containing 0.5 M NaCl. The enzyme was recovered between 25 and 80 ml of the eluate. Another 8-fold purification was achieved by this step with very little loss of activity.

A typical purification is shown in Table I. There is about 1000-fold purification with about 70% recovery of the units. Considerable nucleic acid is removed by this purification as indicated by $A_{280:260}$ absorption ratio (Table I). For most of the studies recorded in this paper the eluate from the second ion exchange column was directly used although the enzyme solution could be concentrated with some loss of activity by putting it in a dialyzing bag and keeping the bag in solid sucrose for 12 to 15 hours. Other procedures for concentration, such as lyophilization, ammonium sulfate concentration, etc. led to 90 to 95% loss of activity. The finally purified enzyme was, however, not homogeneous as shown by polyacrylamide gel electrophoresis. There was one major protein band accompanied with two minor bands.

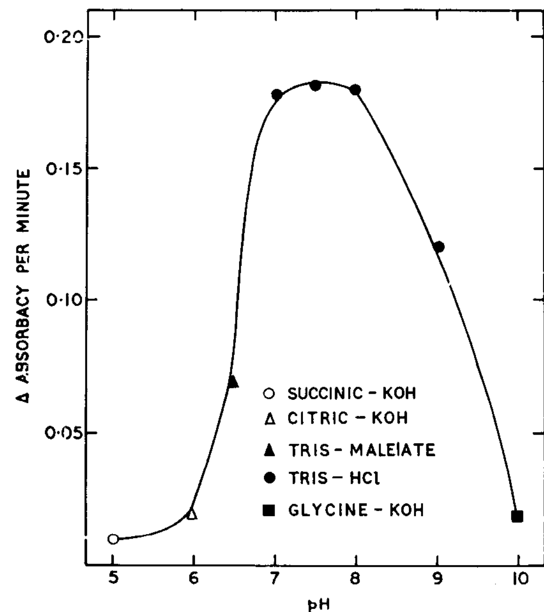

FIG. 3. Effect of pH of the incubation medium on the activity of P22 lysozyme. The enzyme assay was carried out as described under "Materials and Methods," except that the nature and pH of the buffering system were varied as indicated. Purified enzyme (0.1 μg of protein) was used in each assay.

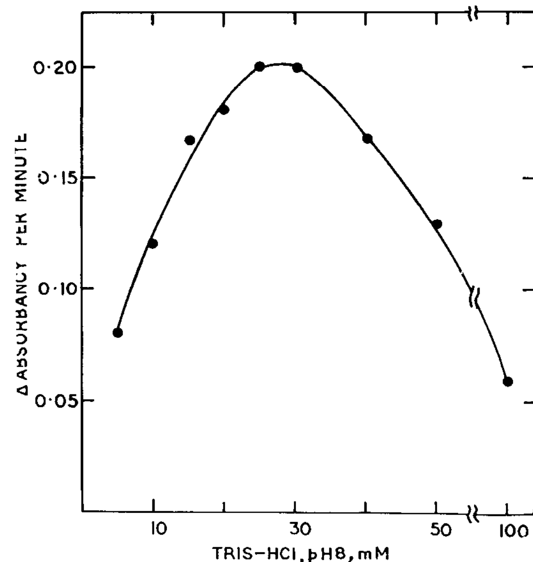

FIG. 4. Effect of ionic strength of the incubation medium on the activity of P22 lysozyme. The enzyme assay was carried out as described under "Materials and Methods," except that the concentration of Tris in the incubation mixture was varied. Purified enzyme (0.07 μg of protein) was used in each assay.

Effect of pH of Incubation Medium on Lysozyme Activity—A number of buffers were used to study the effect of varying pH on the activity of P22 lysozyme (Fig. 3). The enzyme has optimum pH between 7 and 8. At slightly acidic pH (pH 6) there is very little enzyme activity. Although the activity at pH 9 is not too low, at pH 10 there is practically negligible activity. For routine assay pH 8 was chosen. Because of the inhibitory effects of the salts on the lysozyme (results to be presented later) the values presented in Fig. 3 are somewhat less than the optimal

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TABLE II

Substrate specificity of P22 and egg white lysozymes

Enzyme	Substrate	Amount of enzyme in reaction[a]	Unit	Ratio of P22 lysozyme to egg white lysozyme[b]
		μg		
P22 lysozyme	*S. typhimurium* (chloroform-treated cells)	0.04	0.12	200
Egg white lysozyme	*S. typhimurium* (chloroform-treated cells)	4.00	0.06	
P22 lysozyme	*E. coli* B (chloroform-treated cells)	0.04	0.14	233
Egg white lysozyme	*E. coli* B (chloroform-treated cells)	4.00	0.06	
P22 lysozyme	*M. lysodeikticus* (untreated cells)	1.00	0.15	12.5
Egg white lysozyme	*M. lysodeikticus* (untreated cells)	10.00	0.12	

[a] The assay was carried under standard incubation conditions as described in the text. Protein was measured by the Folin reagent as described by Lowry *et al.* (12).

[b] Ratios are calculated on the basis of unit weights of the enzymes. Since the P22 enzyme was not homogeneous its actual weight would be somewhat less than that stated. The method of assay has been described under "Materials and Methods."

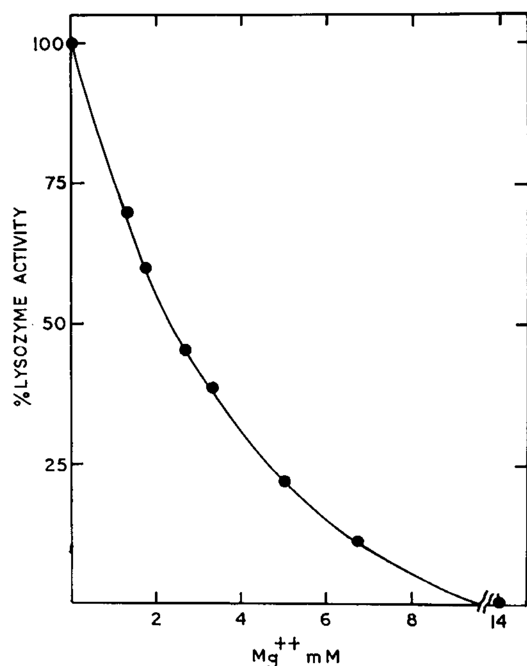

FIG. 5. Effect of varying concentration of $MgCl_2$ on the activity of P22 lysozyme. The enzyme assay was carried out as described under "Materials and Methods" with the addition of varying amount of $MgCl_2$ as indicated.

values. The activity profile against pH, however, does not seem to be much affected by slightly varying salt concentration.

Effect of Ionic Strength of Buffer on Activity of Lysozyme—As discussed above, the optimum pH of the enzyme was between 7 and 8; the pH of 8 was chosen for the routine assay and the buffer chosen was Tris. Since the activity of egg white lysozyme is known to be dependent on the ionic strength of incubation medium (11) the effect of varying concentrations of the buffer on the activity of the P22 lysozyme was tested. The results are presented in Fig. 4. Increasing the concentration of the buffer (Tris) from 0.005 M to about 0.025 M resulted in the gradual increase of the activity. However, increasing the concentration beyond 0.03 M resulted in the lowering of the activity. At low (0.005 M) and high (0.1 M) ionic strength the activity was almost one-third or one-fourth of the maximum activity observed. This clearly indicates that the lysozyme activity is very much dependent on the concentration of the ions present in the incubation medium. For the routine assay a concentration of 0.025 M of Tris was chosen.

Substrate Specificity of P22 Lysozyme—Chloroform-treated *S. typhimurium* cells were used as the substrate for the routine assay of the lysozyme. In order to check its substrate specificity,

TABLE III

Effect of NaCl and KCl on activity of P22 lysozyme

The enzyme assay was carried out as described under "Materials and Methods." Varying amount of salt as indicated was included in the incubation mixture.

Concentration	Residual activity	
	NaCl	KCl
M	%	%
	100	100
0.017	77	93
0.033	53	75
0.051	32	61
0.067	18	50
0.100	10	28
0.133	6	14

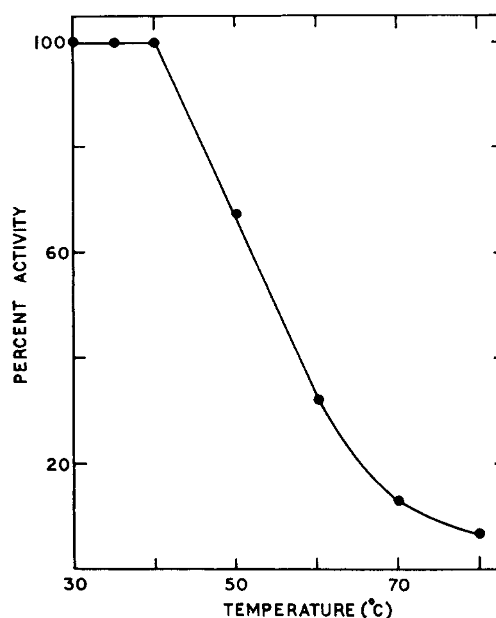

FIG. 6. Thermostability of P22 lysozyme. The purified enzyme (0.5 ml) was incubated for 5 min at the indicated temperatures. Then the solution was cooled in ice and the residual activity was measured by the standard method of assay. Activities are expressed as percentages of the activity of unincubated enzyme.

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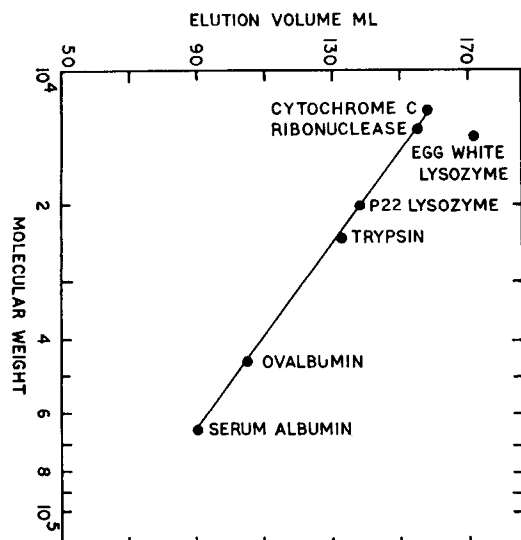

FIG. 7. Molecular weight determination of P22 lysozyme by gel filtration through Sephadex G-100. A column of Sephadex G-100 (2.4 × 53 cm) was used after equilibration with 0.05 M Tris, pH 7.4, containing 0.1 M KCl. The elution was carried out with the same mixture. The void volume was 60 ml. The elution of phage P22 lysozyme was followed by measuring enzyme activity as per standard assay method described in the text. In the case of other proteins absorbancy at 280 nm was measured in a Zeiss ultraviolet spectrophotometer.

TABLE IV

Comparison of properties of three phage-specific lysozymes

Property	T4 (4)	λ (5)	P22
1. Molecular weight	18 to 20 × 10^3	18 × 10^3	20 × 10^3
2. Optimum pH	7	7	7–8
3. Loss of activity			
a. At 50° for 5 min	40%		32%
b. At 60° for 5 min	80%		68%
4. Activity with *E. coli* cells (with respect to egg white lysozyme taken as unity.)	250	10	233
5. Activity with *M. lysodeikticus* cells (with respect to egg white lysozyme taken as unity.)	6.3	0.001	12.5

chloroform-treated *E. coli* cells were also used in similar assay system. Untreated cells of the gram-positive microorganism *M. lysodeikticus* were used for comparative purposes. The activity of the P22 lysozyme with the different substrates was also compared with that of egg white lysozyme. The results have been presented in Table II. It is evident from the results that the gram-negative cells act as better substrates with both phage P22 and egg white lysozymes. The egg white lysozyme was, however, much less active than P22 lysozyme. With gram-negative cells as the substrate the activity of P22 lysozyme was 200 to 250 times greater than that of the egg white lysozyme. The difference, however, becomes comparatively much less (only 12 times) when *M. lysodeikticus* cells are used as the substrate. Since P22 lysozyme was not homogeneous the comparison is somewhat arbitrary.

Effect of $MgCl_2$ on Activity of Lysozyme—The presence of $MgCl_2$ in the incubation medium was inhibitory to lysozyme activity (Fig. 5). Therefore the effect of varying concentration of $MgCl_2$ on the activity of phage P22 lysozyme was studied. There was gradually decreasing activity with the increasing concentration of $MgCl_2$ and the complete inhibition was obtained at a $MgCl_2$ concentration of about 1.0 to 1.4 × 10^{-2} M. Similar inhibition was obtained in presence of $MnCl_2$ (results not presented).

Effect of NaCl and KCl on Lysozyme Activity—Both NaCl and KCl were found to be inhibitory to the lysozyme. The effects of varying concentration of NaCl and KCl were studied and the results are presented in Table III. The inhibition is dependent on the concentration and nature of the salt used. NaCl appears to be more effective than KCl when equimolar concentrations of the two salts are used. The reason for this difference is not known. At a salt concentration of about 0.13 M 90 to 95% inhibition of the lysozyme is observed. The inhibitory effect of $MgCl_2$ can not be considered similar to that of NaCl and KCl as comparatively lower concentration of $MgCl_2$ produces a considerable amount of inhibition (Fig. 5). It has already been mentioned, while presenting results on the effect of pH, that the salt used in preparation of the buffer might influence the enzymatic activity. Thus the amount of the main buffering agent used (*e.g.* Tris in Fig. 4) has to be taken into account to determine the actual rate of the enzyme-catalyzed reaction under different conditions.

Effect of Temperature on Stability of Lysozyme—The enzyme was stable at room temperature but rapidly lost its activity at higher temperatures. Therefore the effect of temperature on the stability of the enzyme was studied. As mentioned in the legend to Fig. 6, the enzyme solution was incubated at various temperatures for 5 min and then assayed under standard conditions at 25°. Incubation for short period at temperatures up to 40° did not result in any loss of activity but incubation at temperatures higher than that led to rapid loss of activity. For example, incubation at 50° leads to loss of more than 30% of the activity. At 70° only 10% of the original activity remained.

In the heat inactivation experiment (Fig. 6), the enzyme preparation was in 0.1 M phosphate, pH 6.5–0.5 M NaCl. In order to check whether high salt concentration may be affecting the results, this enzyme preparation was passed through a Sephadex G-25 column by which the concentrations of phosphate and NaCl were lowered to 0.04 and 0.2 M, respectively. With this preparation almost the same thermostability curve was obtained as shown in Fig. 6.

Determination of Molecular Weight of Lysozyme—Although the enzyme was not obtained in homogeneous state, an attempt was made to estimate the molecular weight by gel filtration through Sephadex G-100 (13). A number of standard markers were used for the purpose and, as indicated in Fig. 7, the elution volume was in perfect linear relationship with the logarithms of the molecular weights of the standard protein markers with one exception. The known molecular weight of the egg white lysozyme did not fit the linear relationship. As pointed out by one of the referees, egg white lysozyme which is highly basic may interact with negative charges present in small amounts on Sephadex. This was probably of no concern in the case of P22 lysozyme which appeared much less basic than egg white lysozyme from its behavior on the phosphocellulose column. Egg white lysozyme is eluted from this column at a concentration of 0.3 M of phosphate, pH 7.5, whereas phage P22 lysozyme is eluted by the same buffer at a concentration of 0.15 M. Phage P22 lysozyme

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was eluted close to trypsin from a Sephadex G-100 column, indicating a molecular weight of about 2×10^4.

DISCUSSION

Although phage P22 lysozyme has been purified extensively (about 1000-fold) starting from the lysate of C_1 infected *S. typhimurium* (Table I) it has not been obtained in a homogeneous state. Polyacrylamide gel electrophoresis indicated the presence of two to three protein bands. Nevertheless, some of the properties of the enzyme could be studied and compared with those of the lysozymes induced in *E. coli* by T4 and λ (Table IV). It has already been mentioned in the introduction that the latter enzymes have been obtained in homogeneous state. The molecular weights of all the three enzymes are in the same range. The optimum pH also seems to be in the same range though P22 lysozyme has a somewhat broad pH optimum. Thermostability of λ lysozyme was not determined but the thermostability of P22 lysozyme is not too different from that of T4 lysozyme. P22 lysozyme seems to be slightly less stable but considering the fact that this lysozyme was not obtained in pure form the comparison is not quite valid. In their action on gram-positive and gram-negative organisms dramatic differences and similarities were observed. The activities have been expressed taking egg white lysozyme activity as unity. For example, the activities of T4 and P22 lysozymes are comparable when gram-negative *E. coli* cells are used as substrate (Table IV). Both of them display lower activities in the same range with gram-positive *M. lysodeikticus* cells which were used without any chloroform treatment. λ Lysozyme has much lower activity with either of the two substrates and its activity is much lower (one-thousandth) with *M. lysodeikticus* than with *E. coli*. This clearly indicates that P22 lysozyme behaves more closely to T4 lysozyme, although the former is a temperate phage and the latter, a virulent one. This reminds one of the structural similarity between P22 and T4 genomes and the dissimilarities in this respect between the two temperate phages, λ and P22. Weak serological cross-reaction has been observed between T4 and λ lysozymes (6). It would be of great interest to determine the serological cross-reaction of P22 lysozyme, if obtained in homogeneous form, with T4 lysozyme.

The egg white lysozyme has been extensively studied and its structural features have been determined (14). The activity of the enzyme is very much dependent on the ionic strength of the incubation medium (11). An initial increase in lytic activity and a subsequent inhibition of activity with increase of ionic strength were more marked at higher pH values than at low ones. The same was found to be true for P22 lysozyme. Increase in concentration of Tris from 1×10^{-2} to 3×10^{-2} M led to increase in lytic activity but further increase in concentration resulted in the lowering of the activity (Fig. 4). Inhibitory effects of NaCl and KCl (Table III) and probably that of $MgCl_2$ (Fig. 5) are caused by the increased ionic strength. As it is true for other phage-specific lysozymes, P22 lysozyme is much more effective than the egg white enzyme, against bacterial cell walls. The difference is more prominent in case of gram-negative cells (Table II).

Although the assay method used for the detection of lysozyme was not too sensitive to detect traces of enzyme activity it is quite evident that P22 lysozyme is induced at the late stage following infection (Fig. 1). This is also supported by the fact that the C_1 gene controls the expression of late genes of P22 and in a C_2 mutant (where C_1 gene is intact) the lysozyme induction is delayed further and the amount of lysozyme produced is considerably less than that produced following infection with C_1.[1]

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