Purification and Properties of a Thermophilic Bacteriophage Lytic Enzyme¹

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A phage lytic enzyme was isolated from lysates of *Bacillus stearothermophilus* (NCA 1503-4R). The enzyme was purified 1,998-fold with a 27% recovery of enzyme activity. By use of polyacrylamide gel electrophoresis and sucrose gradient centrifugation the enzyme was judged free from protein contaminants. The lytic enzyme was active over a pH range of 6.0 to 7.0, with a maximum at 6.3, and it was stable between pH 7.0 and 8.0 and at 5.0 and unstable between pH 5.5 and 6.5. The temperature coefficient (Q_{10}) was 2.27 between 35 and 45 C, 2.01 between 45 and 55 C, and 2.00 between 50 and 60 C. Lytic enzyme in 0.1 M sodium phosphate was not inactivated after a 1-hr exposure to temperatures below 65.5 C, whereas a 1% inactivation was observed at 70.6 C. A 2-hr exposure at 60.1, 65.5, and 70.6 C resulted in an inactivation of 1.2, 9.6, and 12.0%, respectively. A sodium phosphate concentration of at least 0.1 M was necessary for the prolonged exposure of lytic enzyme at 55 C (pH 6.3), whereas 0.005 M was required for maximal lytic activity. Lytic activity was stimulated 169, 165, and 160% by 10^{-4} M Mg^{++} , Ca^{++} , and Mn^{++} , respectively. Lytic activity was inhibited 75% by 10^{-4} M ethylenediaminetetraacetic acid (EDTA). The EDTA inhibition could be reversed by the addition of excess Mg^{++} , Ca^{++} , or Mn^{++} . Lytic activity was not affected by NaCl, KCl, or NH_4Cl . Lytic activity was inhibited 100, 91, 25, 61, and 56% by 10^{-4} M Hg^{++} , Cu^{++} , Zn^{++} , *p*-chloromercuribenzoate, and *p*-hydroxymercuribenzoate, respectively. Cysteine or 2-mercaptoethanol did not stimulate lytic activity, nor were these sulfhydryl compounds required for maintenance of enzyme activity during handling or storage. Cell walls were rapidly solubilized when incubated with lytic enzyme. Lytic action was complete after 1.5 min, with a 70% reduction in optical density (OD). Cell walls without lytic enzyme showed no reduction in OD during this period. The solubilization of N-terminal amino groups paralleled the reduction in OD and reached a level of 0.3 μ mole/mg of cell wall after 4 min of incubation. Cell walls with and without lytic enzyme treatment showed a 3- and a 1.3-fold increase, respectively, in N-terminal amino groups after 3 hr of incubation. There was no release of reducing power in either the untreated cell wall suspensions or those treated with lytic enzyme. Electron micrographs of treated and untreated cell walls showed that the enzyme partially degrades the cell wall with the release of small wall fragments.

Phage reproduction and release of phage particles are usually accompanied by lysis of the bacterial host cell. In gram-positive bacteria, cellular lysis is caused by the breakdown of the rigid cell wall peptidoglycan by a soluble phage-induced enzyme(s). Phage-induced enzymes are found in induced lysogenic or phage-infected cells and are absent in noninduced or uninfected cells (18). Phage-induced enzymes that lyse bacterial cells have been demonstrated in lysates of various phage-host systems and have been designated as

lysozymes (12, 19, 21), muralysins (14; S. S. Barkulis et al., *Bacteriol. Proc.*, p. 32, 1964), virolysins (16), endolysins (6, 7), lytic enzymes (4, 13; P. F. Bartell, T. E. Orr, and W. S. Blake-more, *Bacteriol. Proc.*, p. 142, 1964; J. M. Goepfert and H. B. Naylor, *Bacteriol. Proc.*, p. 142, 1964), or lysins (3, 8, 17). With the exception of lysozyme (endoacetylmuramidase) from phage T2 of *Escherichia coli* B (10, 21) and muralysin (endoacetylglucosamidase) from phage C-1 of group C streptococci (1; S. S. Barkulis et al., *Bacteriol. Proc.*, p. 32, 1964), the information has been mainly descriptive in nature. Welker and Campbell (22; *Bacteriol. Proc.*, p. 126, 1966)

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reported that mitomycin C-induced cultures of *Bacillus stearothermophilus* (NCA 1503-4R) contained considerable quantities of a lytic enzyme which lysed several strains of *B. stearothermophilus*. This paper describes the purification and some of the general properties of this thermophilic phage-induced lytic enzyme.

MATERIALS AND METHODS

The organism used in this study was an obligately thermophilic strain of *B. stearothermophilus* (NCA 1503-4R) which was shown by Welker and Campbell (22) to be carrying bacteriophage TP-1.

Preparation of substrates for lytic enzyme. Acetone-dried cells of *B. stearothermophilus* (NCA 1503-4R) were prepared by use of the procedure described by Work (23). Mid-log-phase cells (optical density at 525 m μ , 0.45) of a culture grown in Trypticase (BBL)-yeast extract-fructose (TYF) were sedimented by centrifugation, washed three times with distilled water, and suspended in sufficient distilled water to make a 70% suspension (w/v). The cell suspension was slowly added with vigorous stirring to 20 volumes of acetone and held at ambient room temperature for 48 hr. The acetone-treated cells were removed by centrifugation, suspended in 10 to 15 ml of distilled water, lyophilized, and stored over NaOH pellets in a vacuum desiccator.

Cell walls of *B. stearothermophilus* (NCA 1503-4R) were prepared as described by Sutow and Welker (20).

Lytic enzyme assay. Lytic activity was measured in a Gilford model 2000 automatic spectrophotometer equipped with dual thermospacers. The assay temperature was maintained with a Haake model F constant-temperature circulator. The assay cuvette contained 2.7 ml of 0.005 M sodium phosphate- 10^{-4} M MgCl₂ buffer, pH 6.3 (PM-buffer), and 0.2 ml of a suspension of acetone-dried cells or cell walls (3.8 mg per ml of PM-buffer). The initial optical density (OD) at 420 m μ for acetone-dried cells and cell walls was 0.75 to 0.90 and 0.50 to 0.65, respectively. After equilibration at 55 C, lytic enzyme (1.1 to 2.0 μ g per 0.1 ml) was added. A unit of enzyme activity is defined as that concentration of lytic enzyme which causes a decrease in OD of 0.1 between 0.5 and 1.5 min after addition of enzyme. Enzyme protein was determined by the method of Lowry et al. (9) by use of bovine serum albumin (Mann Research Laboratories, New York, N.Y.) as a standard.

Sucrose gradient centrifugation. Sucrose gradient centrifugation was carried out by use of the technique described by Martin and Ames (11). Gradients (4.8 ml) from 5 to 20% sucrose (w/v) in 0.005 M sodium phosphate buffer (pH 7.0) were prepared. Purified lytic enzyme (278 μ g) was layered on top of the gradient, and centrifugation was carried out in a Spinco model L ultracentrifuge at 39,000 rev/min (SW-39 rotor) for 22 hr at 3 C. After centrifugation, the bottom of the tube was punctured with a no. 21 hypodermic needle and 7-drop fractions were collected in 11 by 100 mm tubes containing 1 ml of PM-buffer. The absorbancy at 280 m μ and lytic enzyme activity were measured in each fraction.

Polyacrylamide gel electrophoresis. Electrophoresis on polyacrylamide gels was performed as described by Davis (2). The upper gel contained 2.5% acrylamide and 0.6% bisacrylamide, and the lower gel contained 7% acrylamide and 0.18% bisacrylamide. Lytic enzyme (30 μ g) in 10% sucrose was layered over the upper gel, and duplicate gels were run at 3 ma per column in 2.5×10^{-3} M tris(hydroxymethyl)amino-methane (Tris)- 1.9×10^{-2} M glycine buffer (pH 8.6) until the bromophenol blue marker reached the end of the gels (ca. 1 hr). One gel was stained with aniline blue-black, and the other was cut into 2-mm segments. Each segment was placed into a test tube containing 0.5 ml of PM-buffer and held at 5 C for 16 to 20 hr. Protein and lytic enzyme activity was measured in each segment.

Adsorbents. CM-cellulose (standard capacity, 0.7 ± 0.1 meq/g) and diethylaminoethyl (DEAE)-cellulose (standard capacity, 0.9 ± 0.1 meq/g) were obtained from Mann Research Laboratories. Suspensions of DEAE-cellulose or CM-cellulose were prepared by mixing 50 g of the dry powder with 2 liters of 1 M NaOH. After stirring for 30 min, the suspensions were allowed to settle for 30 min, and the fines were removed by decantation. CM- and DEAE-cellulose were suspended in 2 liters of 0.5 M NaCl and distilled water, respectively, and stirred for 30 min. After standing for 30 min, the fines were decanted and the washing procedure was repeated two more times. The CM- and DEAE-cellulose suspensions were exhaustively washed with 0.05 M sodium acetate buffer (pH 5.6) and 0.01 M Tris chloride (pH 8.0), respectively. Chromatographic columns (2.5 cm in diameter) fitted with sintered-glass discs were packed by gravity flow at 5 C to heights of 12.5 and 5.0 cm with CM- and DEAE-cellulose, respectively. The columns were equilibrated with the appropriate buffer and allowed to settle for 24 hr.

Electron microscopy. Cell walls in PM-buffer were treated with lytic enzyme for 1.5 min at 55 C. The walls were removed by centrifugation at $27,000 \times g$ for 30 min and were suspended in 0.5 ml of distilled water. A control, consisting of cell walls not treated with lytic enzyme, was prepared in an identical manner. Droplets of each suspension were placed on Formvar-coated copper grids, air-dried, and shadowed with Nichrome. The grids were examined in a Hitachi HS-7 electron microscope at an initial magnification of $7,500 \times$.

Characterization of lytic activity on cell walls. Cell walls (5.32 mg) were incubated with lytic enzyme (20 μ g) in 21 ml of PM-buffer at 55 C. In the cell wall control, PM-buffer was added in place of enzyme. Duplicate samples (1.0 ml) were removed periodically and placed into 13 by 100 mm glass-stoppered tubes containing 0.25 ml of 1% Na₂B₄O₇·10H₂O or 0.5 ml of 0.53% Na₂CO₃-0.065% KCN solution for the determination of N-terminal amino groups (5) and reducing power (15), respectively. The tubes were placed in an ice bath until all samples were collected. An 18- μ liter amount of 0.1 M 2,4-dinitrofluorobenzene in 100% ethyl alcohol was added for the determination of amino groups. After incubation in a 60 C water bath for 30 min, the samples were cooled in an ice bath and

TABLE 1. *Purification of phage lytic enzyme*

Step	Units per ml ^a	Total units (×10 ³)	Protein	Specific activity (units per mg of protein)	Purification	Yield
			mg/ml		fold	%
Crude lysate.....	86	2,541	8.62	9.9	1	100
First ammonium sulfate fractionation....	3,220	2,528	16.80	191.7	194	99
CM-cellulose adsorption and elution.....	7,720	1,814	1.68	4,595.2	464	71
DEAE-cellulose purification.....	3,040	1,003	0.32	9,500.0	960	39
Second ammonium sulfate fractionation..	55,000	686	2.78	19,784.1	1,998	27

^a A unit of lytic enzyme activity is defined as a decrease in optical density of 0.1 at 420 mμ of a suspension of acetone-dried *Bacillus stearothermophilus* (NCA 1503-4R) cells at 55 C, pH 6.3.

acidified with 1.0 ml of 2 N HCl. The precipitate in each sample was removed by centrifugation at $1,000 \times g$ for 10 min, and the absorbancy at 420 mμ was measured in the Gilford spectrophotometer. Reducing power was measured as follows. After 0.5 ml of 0.05% $K_3Fe(CN)_6$ was added to each sample, the tubes were placed in a boiling-water bath for 15 min. After cooling in an ice bath, 2.5 ml of 0.15% $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ -0.1% sodium docetyl sulfate in 0.15 N H_2SO_4 (5) was added to each tube and the color was allowed to develop for 15 min. The absorbancy at 690 mμ was measured in the Gilford spectrophotometer. Free amino groups and reducing power were expressed relative to standard D-glutamic acid and N-acetylglucosamine (Mann Research Laboratories), respectively.

Preparation of lysate. Each of three 14-liter fermentor vessels containing 12 liters of TYF medium were inoculated with 1 liter of cell inoculum prepared as follows. One Fernbach flask containing 1 liter of TYF medium was inoculated with 12- to 15-hr cells from a Trypticase agar plate and shaken on a New Brunswick model G-25 gyrotory incubator shaker for 2 hr at 55 C. The fermentor vessels were agitated (300 rev/min) and aerated (5,000 cc of filter-sterilized air per min) at 55 C in a New Brunswick fermentor (model FS-314). Growth was measured in a Bausch & Lomb Spectronic-20 colorimeter at 525 mμ. The cultures were treated with mitomycin C (0.05 μg/ml) as described by Welker and Campbell (22). After lysis was complete (usually 60 to 90 min after addition of mitomycin C), the lysates were cooled to 40 C.

RESULTS AND DISCUSSION

Purification of lytic enzyme. The lytic enzyme was purified from 35- to 40-liter quantities of crude lysate by the following procedure.

Step 1: first ammonium sulfate fractionation. The crude lysate was treated with 0.5 μg of deoxyribonuclease (Mann Research Laboratories) per ml for 30 min. The pH was adjusted to 6.0 with concentrated HCl, and 390 g of solid ammonium sulfate per liter was slowly added with stirring. After addition of the ammonium sulfate, the mixture was stirred for 1 hr and placed at 5 C for 4 to 5 days so that the precipitate could settle to

the bottom of the container. The supernatant fluids were decanted, and the precipitate was collected by centrifugation at $16,300 \times g$ for 20 min. The precipitate was suspended in (400 to 600 ml), and dialyzed exhaustively against, 0.01 M sodium phosphate buffer (pH 7.2). The insoluble material was removed by centrifugation at $27,000 \times g$ for 20 min.

Step 2: CM-cellulose adsorption and elution. The enzyme was adjusted to pH 5.6 with 0.35 N acetic acid, and the lytic enzyme was applied to a column of CM-cellulose. The column was washed exhaustively with 0.05 M sodium acetate buffer (pH 5.6) until all the colored material was removed. The lytic enzyme was eluted with 0.05 M sodium acetate buffer (pH 5.6) plus 0.2 M NaCl.

Step 3: DEAE-cellulose purification. The straw-colored enzyme solution was dialyzed for 48 hr at 5 C against 14 liters of 0.01 M Tris chloride buffer (pH 7.0). The pH was adjusted to 8.0 with 1 N HCl, and the enzyme solution was passed through a column of DEAE-cellulose. The eluate, containing the lytic enzyme, was adjusted to pH 6.5 with 1 M NaOH.

Step 4: second ammonium sulfate fractionation. Solid ammonium sulfate (351 g per liter) was slowly added with stirring at 5 C. After standing for 24 to 48 hr, the precipitate was removed by centrifugation at $27,000 \times g$ for 20 min and discarded. Additional solid ammonium sulfate (210 g per liter of original solution) was slowly added with stirring, and the mixture was held at 5 C for 24 hr. The precipitate was removed by centrifugation at $27,000 \times g$ for 10 min, suspended in 5 to 20 ml of a 50% ammonium sulfate solution (w/v), and held at 5 C for 10 to 12 hr. The precipitate was removed by centrifugation at $27,000 \times g$ for 20 min, and the same procedure was repeated with a 45 and a 40% ammonium sulfate solution (w/v). The 40% ammonium sulfate extract contained 95% of the lytic enzyme activity and was dialyzed exhaustively at 5 C against 0.1 M sodium phosphate buffer (pH 7.3). Table 1 summa-

rizates the results of a purification procedure in which the lytic enzyme was purified 1,998-fold with a 27% recovery of lytic enzyme activity. By use of the bacteriophage assay described by Welker and Campbell (22), the purified lytic enzyme was found to be free from phage TP-1.

Polyacrylamide gel electrophoresis of the lytic enzyme revealed a single protein staining band (Fig. 1a). A duplicate gel that was cut into 2-mm segments showed a single protein band which coincided with lytic enzyme activity (Fig. 1b). Sedimentation of the lytic enzyme through a sucrose gradient showed that lytic enzyme activity and the protein boundary sedimented together (Fig. 2). The specific activity in fractions 25 to 35 was between 18,900 and 19,100 units per mg. These results indicated that the lytic enzyme preparation was free from protein contamination and was suitable for a study of some of its general properties.

Effect of pH on lytic enzyme activity and stability. The effect of pH on lytic enzyme activity

FIG. 1. Electrophoresis of purified lytic enzyme on polyacrylamide gel. (a) Gels were stained with aniline blue-black. Control, left; lytic enzyme (30 μ g), right. (b) A duplicate gel was cut into 2-mm segments. Protein and lytic activity were measured in each segment.

FIG. 2. Sucrose gradient centrifugation of purified lytic enzyme. Symbols: $\circ$, lytic enzyme activity; $\bullet$, absorbancy at 280 $m\mu$.

FIG. 3. Effect of pH on lytic enzyme activity. Assays were carried out at 55 C. Symbols: $\circ$, universal buffer; $\bullet$, 0.005 M sodium phosphate buffer.

was determined by the use of acetone-dried cells suspended in 0.005 M sodium phosphate buffer (pH 6.0 to 6.5) and a universal buffer (pH 5.0 to 8.5) prepared as follows. A 10-ml amount of 0.2 M boric acid, 0.2 M acetic acid, and 0.2 M Na_2HPO_4 was added to 120 ml of distilled water. The buffer was adjusted to the desired pH with 1 N NaOH and brought to 200 ml with distilled water. The concentration of each component in the buffer was 0.01 M . The lytic enzyme was active over a pH range of 6.0 to 7.0, with a maximum at 6.3 (Fig. 3). There was no difference in the activity of the lytic enzyme in the presence of the two buffers. The stability of the lytic enzyme as a function of pH was determined by exposing lytic enzyme (98 μ g/ml) to buffers at various pH values for 2 hr. Samples were diluted with PM-buffer and assayed for lytic activity. The lytic enzyme was stable be-

FIG. 4. Effect of pH on the stability of purified lytic enzyme. Lytic enzyme was assayed in PM-buffer at 55 C. Symbols: ▲, universal buffer; △, 0.03 M sodium acetate buffer; ●, 0.03 M sodium phosphate buffer; ○, 0.03 M Tris chloride buffer.

FIG. 5. Effect of temperature on lytic enzyme activity. Lytic enzyme was assayed in PM-buffer.

tween pH 7.0 and 8.0 and at 5.0, and unstable between pH 5.5 and 6.5 (Fig. 4). At pH 6.0, the lytic enzyme in 0.03 M sodium phosphate buffer or universal buffer was inactivated by 8 and 18%, respectively. Inactivation of the lytic enzyme was more pronounced with the universal buffer.

Effect of temperature on lytic enzyme activity and stability. Lytic enzyme activity was determined over a temperature range of 20 to 85 C (Fig. 5). The temperature coefficient (Q_{10}) was 2.27 between 35 and 45 C, 2.01 between 45 and 55 C, and 2.00 between 50 and 60 C. The stability of the lytic enzyme with respect to temperature was determined by exposing samples of lytic enzyme (120 μ g/ml) in 0.005 or 0.1 M sodium phosphate at various temperatures. Samples were removed at 1- and 2-hr intervals, diluted with PM-buffer, and assayed for lytic activity. Lytic

TABLE 2. Thermostability of lytic enzyme^a

Temp	Percentage inactivation			
	0.005 M		0.1 M	
	1 hr	2 hr	1 hr	2 hr
C				
55.1	8.0	15.0	0	0
60.1	12.0	25.8	0	1.2
65.5	18.7	25.5	0	9.6
70.6	25.7	39.7	1.0	12.0

^a Lytic enzyme (120 μ g/ml), in sodium phosphate buffer (pH 6.3), was exposed for 1 and 2 hr at the indicated temperatures. Lytic enzyme was assayed in PM-buffer at 55 C.

FIG. 6. Effect of sodium phosphate concentration on lytic enzyme activity. Assays were carried out at 55 C, pH 6.3.

enzyme in 0.1 M sodium phosphate was not inactivated after a 1-hr exposure to temperatures below 65.5 C (Table 2), whereas a 1% inactivation was observed at 70.6 C. A 2-hr exposure at 60.1, 65.5, and 70.6 C resulted in a 1.2, 9.6, and 12.0% inactivation, respectively. Lytic enzyme in 0.005 M sodium phosphate was inactivated by 8 and 15% after a 1- and 2-hr exposure, respectively, at 55.1 C. The extent of inactivation increased with increase in temperature, with an inactivation of 25.7 and 39.7% after a 1- and 2-hr exposure, respectively, at 70.6 C. Lytic enzyme was not inactivated when exposed for 20 min to the standard assay conditions described in Materials and Methods. Although 0.1 M sodium phosphate enhances the thermal stability of the enzyme, the data indicate that the phage-induced

TABLE 3. *Effect of sodium phosphate buffer concentration on lytic enzyme stability^a*

Molarity	Inactivation
	%
0.001	25
0.002	17
0.005	15
0.075	10
0.01	5
0.05	3
0.10	0
0.25	0
0.50	0

^a Lytic enzyme (96 $\mu\text{g/ml}$), in sodium phosphate buffer (pH 6.3), was held for 2 hr at 55 C and assayed in PM-buffer.

TABLE 4. *Stimulation of lytic enzyme activity by divalent cations*

Addition	Molarity	Activity ^a (% of control)
MgCl_2	10^{-2}	56
	10^{-4}	169
CaCl_2	10^{-3}	50
	10^{-4}	165
MnCl_2	10^{-3}	49
	10^{-4}	160
BaCl_2	10^{-3}	45
	10^{-4}	120
CoCl_2	10^{-3}	46
	10^{-4}	129

^a Lytic enzyme was assayed in PM-buffer minus MgCl_2 at 55 C.

lytic enzyme is relatively resistant to thermal inactivation. Selection of 55 C as the assay temperature for the lytic enzyme is justified on the basis of enzyme stability as well as being the optimal temperature for its synthesis (Welker, unpublished data).

Effect of buffer concentration on lytic enzyme activity and stability. The effect of sodium phosphate concentration on lytic enzyme activity was determined over the range of 0.001 to 0.02 M. Figure 6 shows that maximal lytic activity occurred with 0.005 M sodium phosphate. The stability of the lytic enzyme with respect to sodium phosphate concentration was determined by exposing samples of lytic enzyme (96 $\mu\text{g/ml}$) in 0.001 to 0.5 M sodium phosphate for 2 hr. Samples were diluted so that the final enzyme concentration and buffer molarity was 1.1 to 1.8 $\mu\text{g/ml}$ and 0.005 M, respectively. The lytic enzyme was not inactivated between 0.1 and 0.5 M sodium phosphate (Table 3). The lytic enzyme was less stable as the molarity of the buffer was decreased from

0.05 to 0.001 M. There was no difference in lytic enzyme activity or stability when buffers were prepared with the mono- and dibasic phosphates of either Na^+ or K^+ . These results indicated that, for prolonged exposure at 55 C (pH 6.3), a sodium phosphate concentration of at least 0.1 M is necessary for lytic enzyme stability, whereas 0.005 M is required for lytic activity.

Effect of divalent cations and EDTA on lytic enzyme activity. The effect of various divalent cations on lytic enzyme activity is shown in Table 4. Lytic enzyme activity was stimulated 169, 165, and 160% by the addition of 10^{-4} M Mg^{++} , Ca^{++} , and Mn^{++} , respectively, whereas 10^{-4} M Ba^{++} and Co^{++} showed only a slight stimulation. Lytic enzyme activity was not affected by Fe^{++} , Cd^{++} , or Ni^{++} . No difference in the stimulation of lytic enzyme activity was observed between the chloride and sulfate salts of the metals. All the divalent cations inhibited lytic enzyme activity at a concentration of 10^{-3} M. The stimulatory effect of Mg^{++} , Ca^{++} , and Mn^{++} on lytic enzyme activity was determined over the range of 10^{-2} to 10^{-6} M (Fig. 7). There was no significant difference in the stimulatory effect of the three cations; however, Mg^{++} was employed in the assay because at 10^{-4} M it gave a slightly higher stimulation of enzyme activity than either Ca^{++} or Mn^{++} . The effect of EDTA on lytic enzyme activity is shown in Table 5. Enzyme activity was inhibited by 13, 69, 75, and 94% by 10^{-5} , 5×10^{-5} , 10^{-4} , and 10^{-3} M EDTA, respectively. The inhibition of lytic enzyme activity with 10^{-4} M EDTA could be reversed by the addition of 10^{-4} M Mg^{++} , whereas excess Mg^{++} (5×10^{-4} M) resulted in a stimulation of lytic activity. Lytic enzyme activity was inhibited 98% after dialysis for 24 hr at 5 C against 2 liters of 0.1 M sodium phosphate buffer (pH 6.3) plus 10^{-3} M EDTA. After removal of the EDTA by dialysis

FIG. 7. Effect of magnesium, calcium, and manganese ions on lytic enzyme activity. Lytic enzyme was assayed in PM-buffer minus MgCl_2 at 55 C. Symbols: $\circ$, Mg^{++} ; $\triangle$, Ca^{++} ; $\bullet$, Mn^{++} .

TABLE 5. Effect of EDTA on lytic enzyme activity

Addition	Activity ^a (% of control)
EDTA, 10 ⁻³ M	6
EDTA, 10 ⁻⁴ M	25
EDTA, 5 × 10 ⁻⁵ M	31
EDTA, 10 ⁻⁵ M	87
EDTA (10 ⁻⁴ M) plus MgCl ₂ , 10 ⁻⁴ M	98
MgCl ₂ , 5 × 10 ⁻⁴ M	130

^a Lytic enzyme was assayed in PM-buffer minus MgCl₂ at 55 C.

TABLE 6. Inhibition of lytic enzyme activity by divalent cations, PHMB, and PCMB

Addition	Molarity	Inhibition ^a
		%
HgCl ₂	10 ⁻⁴	100
	5 × 10 ⁻⁵	75
	10 ⁻⁵	65
CuSO ₄	10 ⁻⁴	91
	5 × 10 ⁻⁵	50
	10 ⁻⁵	20
ZnCl ₂	10 ⁻⁴	25
	5 × 10 ⁻⁵	5
	10 ⁻⁵	0
PHMB	10 ⁻⁴	56
PCMB	5 × 10 ⁻⁵	61
	10 ⁻⁵	11

^a Lytic enzyme was assayed in PM-buffer at 55 C.

against 0.1 M sodium phosphate buffer (pH 6.3), lytic enzyme activity could be restored by addition of 10⁻⁴ M Mg⁺⁺, Ca⁺⁺, or Mn⁺⁺. These results indicated that divalent cations (Mg⁺⁺, Ca⁺⁺, or Mn⁺⁺) are required for lytic enzyme activity. Lytic enzyme activity was not affected by the addition of 10⁻³ to 10⁻⁵ M NaCl, KCl, or NH₄Cl.

Inhibition of lytic enzyme activity by divalent cations, p-hydroxymercuribenzoate (PHMB), and p-chloromercuribenzoate (PCMB). Lytic enzyme activity was inhibited 100, 91, and 25% by the addition of 10⁻⁴ M Hg⁺⁺, Cu⁺⁺, and Zn⁺⁺, respectively (Table 6). At 10⁻⁵ M, Hg⁺⁺ and Cu⁺⁺ inhibited lytic enzyme activity by 65 and 20%, respectively, whereas Zn⁺⁺ was noninhibitory. Lytic enzyme activity was inhibited 56 and 61% by 10⁻⁴ M PHMB and PCMB, respectively. These results indicated that free sulfhydryl groups are necessary for lytic enzyme activity. Cysteine or 2-mercaptoethanol did not stimulate lytic activity, nor were these sulfhydryl compounds required for maintenance of enzyme activity during handling or storage.

Action of lytic enzyme on cell walls. The lytic

FIG. 8. Effect of lytic enzyme concentration on lytic activity. Symbols: ○, acetone-dried cells; ●, cell walls

FIG. 9. Solubilization of cell walls by lytic enzyme. Cell walls (0.75 mg) were incubated with lytic enzyme (1.9 μg) in 3 ml of PM-buffer at 55 C. The optical density at 420 mμ was measured in a Gilford spectrophotometer at 0.5-min intervals. Symbols: ●, cell walls plus lytic enzyme; ○, cell walls.

enzyme assay was valid for amounts of enzyme which resulted in changes in OD of not less than 0.1 and not more than 0.30 to 0.45 during the 1-min assay period. Figure 8 shows the linear de-

pendence of enzyme activity on enzyme concentration when acetone-dried cells or cell walls were used as the substrate. Although the lytic enzyme was more active on acetone-dried cells, cell walls

FIG. 10. Solubilization of N-terminal amino groups from cell walls treated with lytic enzyme. Symbols: ○, N-terminal amino groups; ●, reducing power; solid lines, cell walls plus lytic enzyme; broken lines, cell walls.

were more reliable as a substrate, because there was less variation with cell walls prepared from different batches of cells. Cell walls were rapidly solubilized when incubated with lytic enzyme (Fig. 9). The lytic action was complete after 1.5 min with a 70% reduction in OD, whereas further incubation (3 hr) resulted in an 82% reduction in OD. A suspension of cell walls without lytic enzyme showed no reduction and a 56% reduction in OD after incubation for 1.5 min and 3 hr, respectively. Solubilization of N-terminal amino groups from cell walls incubated with lytic enzyme is shown in Fig. 10. The solubilization of amino groups parallels the reduction in OD (Fig. 9) and reaches a level of 0.3 μ mole/mg of cell wall after 4 min of incubation. This is a threefold increase in the soluble N-terminal amino groups present in untreated cell walls. Cell walls not incubated with lytic enzyme showed a 1.3-fold increase in soluble N-terminal amino groups after 3 hr. An autolytic enzyme localized in the cell walls of *B. stearothermophilus* (NCA 1503-4R) was responsible for the slow release of amino groups (Zarpas and Welker, unpublished data). There was no release of reducing power in lytic enzyme-treated or untreated cell wall suspensions. A comparison of electron micrographs of lytic enzyme-treated (Fig. 11A) and untreated (Fig. 11B) cell wall suspensions shows that the cell wall

FIG. 11. Electron micrographs of lytic enzyme-treated and untreated cell walls. (A) Cell walls, $\times 24,475$. (B) Cell wall incubated with lytic enzyme, $\times 24,475$.

was partially degraded. The termination of lytic activity (Fig. 9) did not correspond to the complete solubilization of the cell wall, but resulted in the release of small wall fragments. The purified lytic enzyme will not release N-terminal amino groups from bovine serum albumin, casein, or gelatin (Mann Research Laboratories).

These results indicated that the phage-induced lytic enzyme is a peptidase which hydrolyzes peptide bonds in the cell wall peptidoglycan. The peptidase exhibits a degree of specificity in that it is active on cell walls from only a small number of *B. stearothermophilus* strains. This is the first example of a phage-induced lytic enzyme, free from all other protein contamination, that has peptidase activity (5). A detailed study of the action of the peptidase on cell wall peptidoglycan will be reported separately. In this connection, the phage peptidase will prove to be a valuable tool in studies on the structure of cell walls of thermophiles.

Although the purified phage-induced peptidase is obtained in relatively small quantities (2.5 to 4.0 mg per 35 liters of lysate), sufficient enzyme is now available so that detailed physical and chemical studies can be carried out to determine which properties are responsible for its intrinsic thermal stability.

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LITERATURE CITED

1. BARKULIS, S. S., C. SMITH, J. J. BOLTRALIK, AND H. HEYMANN. 1964. Structure of streptococcal cell walls. IV. Purification and properties of streptococcal phage muralysin. *J. Biol. Chem.* **239**:4027-4033.
2. DAVIS, B. J. 1964. Disc electrophoresis. II. Method and application to human serum protein. *Ann. N.Y. Acad. Sci.* **121**:404-427.
3. DOUGHTY, C. C., AND J. A. HAYASHI. 1962. Enzymatic properties of a phage-induced lysin affecting group A streptococci. *J. Bacteriol.* **83**:1058-1068.
4. FOX, E. N., AND M. K. WITTNER. 1965. Observations on the group C streptococcal bacteriophage and lytic enzyme system. *J. Bacteriol.* **89**:496-502.
5. GHUYSEN, J. M., D. J. TIPPER, AND J. L. STROMINGER. 1966. Enzymes that degrade bacterial cell walls, p. 685-699. In S. P. Colowick and N. O. Kaplan [ed.], *Methods in enzymology*, vol. 8. Academic Press, Inc., New York.
6. GROMAN, N. B., AND G. SUZUKI. 1962. Relation of endolysin to lysis by lambda bacteriophages. *J. Bacteriol.* **84**:596-597.
7. JACOB, F., AND C. R. FUERST. 1958. The mechanism of lysis by phage studied with defective lysogenic bacteria. *J. Gen. Microbiol.* **18**:518-526.
8. KRAUSE, R. M. 1957. Studies on bacteriophages of hemolytic streptococci. I. Factors influencing the interaction of phage and susceptible host cell. *J. Exptl. Med.* **106**:365-384.
9. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
10. MAASS, D., AND W. WEIDEL. 1963. Final proof for the identity of enzymic specificities of egg-white lysozyme and phage T2 enzyme. *Biochim. Biophys. Acta* **78**:369-370.
11. MARTIN, R. G., AND B. N. AMES. 1961. A method for determining the sedimentation behavior of enzymes: Application to protein mixtures. *J. Biol. Chem.* **236**:1372-1379.
12. MERIGAN, T. C., AND W. J. DREYER. 1963. Studies on the antigenic combining sites in bacteriophage lysozyme. *Ann. N.Y. Acad. Sci.* **103**:765-772.
13. MURPHY, J. S. 1957. A phage-associated enzyme of *Bacillus megaterium* which destroys the bacterial cell wall. *Virology* **4**:563-581.
14. MURPHY, J. S. 1960. An agent derived from *B. megaterium* phage G which dissolves the bacterial cell wall. *Virology* **11**:510-513.
15. PARK, J. T., AND M. J. JOHNSON. 1949. A sub-microdetermination of glucose. *J. Biol. Chem.* **181**:149-151.
16. RALSTON, D. J., B. S. BAER, M. LIEBERMAN, AND A. P. KRUEGER. 1955. Virolysin: A virus-induced lysin from staphylococcal phage lysates. *Proc. Soc. Exptl. Biol. Med.* **89**:502-507.
17. REITER, B., AND J. D. ORAM. 1963. Group N streptococcal phage lysin. *J. Gen. Microbiol.* **32**:29-32.
18. STENT, G. S. 1963. *Molecular biology of bacterial viruses*. W. H. Freeman and Co., San Francisco.
19. STREISINGER, G., F. MUKAI, W. J. DREYER, B. MILLER, AND S. HORIUCHI. 1961. Mutations affecting the lysozyme of phage T4. *Cold Spring Harbor Symp. Quant. Biol.* **16**:25-30.
20. SUTOW, A. B., AND N. E. WELKER. 1967. Chemical composition of the cell walls of *Bacillus stearothermophilus*. *J. Bacteriol.* **93**:1452-1457.
21. WEIDEL, W., AND W. KATZ. 1961. Reindarstellung und charakterisierung des für die lyse T2-infizierter zellen verantwortlichen enzymes. *Z. Naturforsch.* **16b**:156-162.
22. WELKER, N. E., AND L. L. CAMPBELL. 1965. Induction and properties of a temperate bacteriophage from *Bacillus stearothermophilus*. *J. Bacteriol.* **89**:175-184.
23. WORK, E. 1959. The action of a lytic enzyme from spores of a bacillus on various species of bacteria. *Ann. Inst. Pasteur* **96**:486-480.