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Isolation and Characterization of Three Autolytic Enzymes Associated with Sporulation of *Bacillus thuringiensis* var. *thuringiensis*[1]

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Cells of *Bacillus thuringiensis* containing refractile spores autolyzed readily when suspended in buffer. The autolysate contained enzymes which lysed vegetative cell walls of the organism. Three enzymes were isolated from the autolysate, and each was purified approximately 30-fold. One enzyme, most active near *p*H 4.0, was found to be an *N*-acetylmuramidase. The other two enzymes exhibited *p*H optima at 8.5. One was stimulated by cobalt ions and the other was not. The cobalt-stimulated enzyme was shown to be an *N*-acetylmuramyl-L-alanine amidase. The cobalt insensitive enzyme exhibited both *N*-acetylmuramyl-L-alanine amidase and endopeptidase activity. The amidase activity may reflect incomplete separation of the cobalt-stimulated enzyme. The endopeptidase cleaved the peptide bond between L-alanine D-glutamic acid. A cell wall lytic endopeptidase with this specificity has not been previously reported. All three enzymes were extremely limited in the range of bacterial cell walls which they attacked. Except for cell walls of *Micrococcus lysodeikticus*, which were lysed by the muramidase, only cell walls of members of the genus *Bacillus* were attacked.

The release of the mature bacterial spore from its sporangium apparently involves the action of an autolytic enzyme system. Only a few of these enzymes have been investigated, and none has been well characterized. Greenberg and Halvorson (4) found that exponential phase cells of *Bacillus terminalis* (*B. cereus* T) were lysed by supernatant fluids obtained from sporulating cultures of this organism. The lytic activity exhibited a *p*H optimum of 5.0 to 5.5 and was precipitated with ammonium sulfate between 45 and 70% of saturation.

Strange and Dark (11) reported that incubation of sporulating cells of *B. cereus* at 37 C in buffer containing toluene resulted in release from the cells of autolytic enzymes. The crude autolysate, which lysed both vegetative cells and cell walls of the organism, possessed dual *p*H optima near 4.5 and 8.0. Lysis at *p*H 8.0, but not at *p*H 4.5, was markedly stimulated by cobalt ions. Acidification of autolysate to *p*H 3.0 resulted in the precipitation of most of the *p*H 8.0 activity while the *p*H 4.5 activity remained soluble. The mechanisms by which these two fractions lysed cell walls were not investigated. Strange and Dark (10) proposed that the *p*H 8.0 activity might be identical to the lytic system which they found previously in aqueous extracts of disintegrated spores of *B. cereus* and *B. anthracis*.

Autolysates of sporulating *B. thuringiensis* var. *thuringiensis* contain enzymes which lyse cell walls of the organism optimally at *p*H 4.0 and 8.5. We report the separation of the autolytic system into three enzymes and the mechanisms by which each attacks the cell walls.

MATERIALS AND METHODS

Cultivation of cells. B. thuringiensis var. *thuringiensis* Berliner (ATCC 10792) was used for all studies. Cells were grown in a 40-liter stainless-steel fermentor) (Stainless and Steel Products Co., St. Paul, Minn.) containing 30 liters of a peptone medium previously described (6). The inoculum was prepared by a modification of the active culture technique first described by Collier (1). A loopful of material was transferred from an agar slope of completely sporulated cells into 40 ml of medium in a 500-ml Erlenmeyer flask. The flask was shaken at 235 rev/min on a New Brunswick model VS rotary shaker at 30 C. After 9 hr of incubation, 4.0 ml was removed and used to inoculate each of

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three similar flasks containing 40 ml of medium. These were incubated under the same conditions for 2 hr, and the entire content of each was transferred to a 2-liter Erlenmeyer flask containing 500 ml of medium. After 2 hr of shaking at 340 rev/min and at 30 C, the contents of the three large flasks were pooled and aseptically transferred to the precharged fermentor. The temperature of the fermentor was maintained at 30 C, and sterile air was sparged through the medium at approximately 85 liters per min. A pressure of 10 psi was maintained above the medium.

Growth measurements. Turbidity was measured by means of a Bausch & Lomb Spectronic-20 colorimeter at 650 nm. The numbers of total cells, phase dark endospores, phase light endospores, free spores, and parasporal bodies were measured by direct microscopic count using a Petroff-Hauser counting chamber and phase contrast optics.

Preparation of crude autolysate. Cells were grown until 2 to 5% of the total spores present had been released into the medium (usually after about 27 hr of incubation). Crushed ice was added to rapidly cool the culture. The cells were then harvested with a Sharples supercentrifuge yielding 90 to 100 g (wet weight) of packed cells. The paste was rapidly frozen and stored at −16 C. For preparation of lytic enzymes, the cells were thawed and suspended to 10% (w/v) in 0.02 M potassium phosphate buffer, *p*H 7.8. In some instances, where specifically noted, toluene was added to 2.5% (v/v). The suspension was shaken for 2 hr at 30 C on a rotary shaker at 135 rev/min, rapidly cooled, and then centrifuged at 35,000 × *g* for 60 min at 4 C in a refrigerated centrifuge. When toluene was added, it was removed prior to centrifugation by evaporation under a stream of air until no residual odor could be detected. The supernatant fluid contained the active cell wall lytic system.

Enzyme assays. Assays for enzyme activity were based upon the decrease in turbidity accompanying lysis of purified *B. thuringiensis* cell walls. The cell walls were prepared by disruption of exponentially growing cells by sonic oscillation followed by repeated washing, as described previously (6). The enzyme most active at *p*H 4.1 was assayed by adding 50 μliters of enzyme sample to 350 μliters of 0.034 M potassium phthalate-NaOH buffer, *p*H 4.1. Occasionally, a protein precipitate formed which interfered with the assay; when this occurred the precipitate was removed by centrifugation. Then, 200 μliters of cell walls (0.6 mg of cell walls) was added, the suspension was mixed, and the optical density at 650 nm was followed for 10 to 15 min at 37 C in a Cary model 15 recording spectrophotometer. In each assay, the initial optical density of the reaction mixture was approximately 0.5.

The *p*H 8.5 enzyme was assayed by adding 50 μliters of enzyme sample to 350 μliters of a solution containing 0.034 M tris(hydroxymethylaminomethane) Tris chloride buffer, pH 8.5, and 1.7×10^{-4} M $CoCl_2$ to which had just been added 5 μliters of 1.0% (v/v) 2-mercaptoethanol. After 10 min of incubation at 37 C, 200 μliters of cell wall suspension (0.6 mg of cell walls) was added, and the optical density change was monitored as above.

For both assays, 1 unit of activity is defined as that amount of enzyme giving an initial linear decrease in optical density of 0.001 in 5 min. Specific activity is defined as units per milligram of protein.

Column chromatography and gel filtration. Diethylaminoethyl (DEAE)-cellulose (Mann Research Laboratories, Inc., New York, N.Y.) was prepared for use according to the method of Peterson and Sober (9).

Gel filtration was performed on Sephadex G-100 and G-200 (Pharmacia, Inc., Rochester, Minn.), thoroughly equilibrated with the starting buffer.

Chemical assays. Protein was measured by the procedure of Lowry et al. (8) incorporating the modification of Eggstein and Kreutz (2). The standard protein used was egg white lysozyme (Sigma Chemical Co., St. Louis, Mo.).

Total amino acids, N-terminal and C-terminal amino acids, reducing power, and hexosamines were determined as previously described (6).

RESULTS

Microscopic examination of the culture growing in the fermentor revealed that phase dark zones first appeared within the cells after 11 hr of incubation. By 14 hr, forespores were detected in 88% of the population. Between 15 and 24 hr the forespores became phase light, and by 24 hr, 92% of the cells contained phase light spores. Free spores were first observed at 27 hr and by 68 hr the process of spore release was 97% complete. The rather slow release of spores from the sporangia was speeded up considerably when the cells were harvested at the time when spore release had just begun (2 to 5% free spores), then suspended (10% w/v) in 0.02 M potassium phosphate buffer, *p*H 7.0, and shaken at 135 rev/min at 30 C. Under these conditions spore release was complete within 2 hr. Centrifugation yielded a crude autolysate, which rapidly lysed exponential phase cell walls of *B. thuringiensis* suspended in 0.02 M potassium phosphate buffer at *p*H 7.0.

Strange and Dark (11) observed that lysis of cell walls of *B. cereus* by an autolysate of sporulating cells of that organism was highly stimulated by cobalt ions. This prompted an investigation of the effect of various metal ions on the *B. thuringiensis* system. The crude autolysate (10 ml) was dialyzed against 1 liter of 0.02 M Tris-chloride buffer, *p*H 7.5, for 8 hr, followed by a second liter for 16 hr. The preparation was then assayed in the presence of several metal ions at concentrations of 10^{-3} and 10^{-4} M (Table 1). The lytic activity was stimulated approximately 2.5-fold by cobalt ions at 10^{-4} M. At a higher concentration, 10^{-3} M, the Co^{++} stimulation was less. Nickel and zinc ions inhibited the reaction. Calcium, magnesium, and manganese ions stimulated the lytic activity slightly. The effect upon lytic activity of varying the Co^{++} concentration over a wide range

TABLE 1. *Effect of divalent cations on lysis of cell walls by a dialyzed crude autolysate of Bacillus thuringiensis*

Cation added[a]	Relative rate of lysis[b]	
	10^{-3} M	10^{-4} M
None	1.0	1.0
Co^{++}	1.9	2.6
Ca^{++}	1.2	1.0
Ni^{++}	0.2	0.7
Zn^{++}	0.3	0.8
Mg^{++}	1.2	1.1
Mn^{++}	1.3	1.1

[a] The cations were added, at final concentrations of 10^{-3} and 10^{-4} M, as the sulfate salts (Zn^{++}, Mg^{++}, and Mn^{++}) or as the chloride salts (Co^{++}, Ca^{++}, and Ni^{++}).

[b] Reactions contained, in a total volume of 2.0 ml, 100 μliters of autolysate, metal ions and 0.03 M Tris-chloride buffer, *p*H 7.5. After preincubation for 10 min at 37 C, 1.0 ml of cell wall suspension (3.0 mg) was added. The reactions were incubated at 37 C, and the optical density followed at 650 nm for 120 min. The results are presented as a comparison to the rate of lysis with no added cations that was arbitrarily assigned the rate of 1.0.

was determined. Maximal stimulation of activity occurred at approximately 10^{-4} M.

The effect of *p*H on lytic activity in the presence of 10^{-4} M Co^{++} was investigated. Two fairly sharp *p*H optima, one near *p*H 4.0 and the other near *p*H 8.5, were observed (Fig. 1). All subsequent assays were performed in either 0.02 M potassium acid phthalate-NaOH buffer, *p*H 4.1, or 0.02 M Tris-chloride buffer, *p*H 8.5.

The lytic activity at *p*H 8.5, in both dialyzed and undialyzed preparations, was markedly stimulated by cobalt ions, whereas that at *p*H 4.1 was unaffected. The effect on the *p*H 8.5 activity of varying the concentration of Co^{++} is shown in Fig. 2. The lowest concentration tested, 10^{-5} M, markedly stimulated lytic activity as compared to that observed with no added Co^{++}. The optimal level of stimulation occurred between 0.33×10^{-4} and 10^{-4} M. The extent of stimulation decreased with increased Co^{++} concentration until, at 10^{-3} M, there was slightly less activity than in the control with no Co^{++} added. It was necessary to preincubate the enzyme with Co^{++} for at least 10 min prior to adding substrate in order to obtain the maximal stimulatory effect.

Relationship of lytic enzymes to the growth cycle. The time at which the lytic enzymes were produced during growth and sporulation was determined. Samples containing 150 to 250 mg of cells

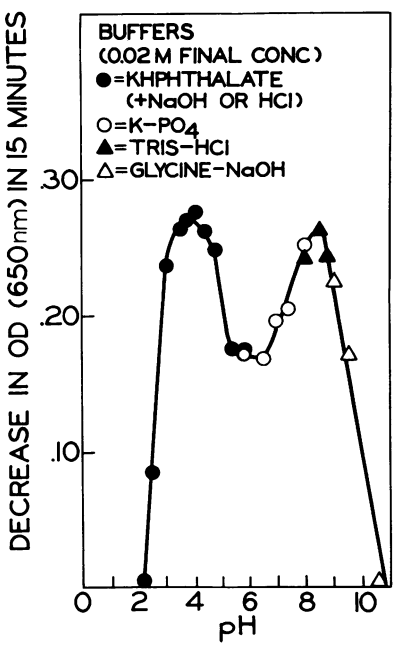

FIG. 1. *Effect of pH on lysis of Bacillus thuringiensis cell walls by a crude autolysate of sporulating cells. The reaction mixtures contained 0.3 ml of 10^{-3} M $CoCl_2$, 1.0 ml of cell wall suspension (1.0 mg), 0.6 ml of 0.1 M buffer, 1.0 ml of water, and 100 μliters of a crude autolysate. The optical density at 650 nm was followed during incubation at 37 C for 15 min.*

(dry weight) were removed from the fermentor at various times, and the cells were sedimented by centrifugation. The cells were suspended in 10 ml of 0.02 M potassium phosphate buffer, *p*H 7.8, disrupted by sonic oscillation, then centrifuged to remove residual intact cells, spores, and debris. The cell-free extracts were assayed for the presence of lytic activity. No lytic activity was found in the extracts until after sporulation had begun (Fig. 3). The intracellular activity then increased, reaching a maximal level at approximately the time when spores began to be released from the sporangia. At this time, progressive lysis of the sporangia resulted in release of free spores and cytoplasmic constituents, with a concomitant drop in the amount of lytic activity in the sonic extracts.

Optimal conditions for release of enzyme from sporulating cells. Sporulating cells were removed by centrifugation from the culture media and suspended to 10% (wet weight/v) in 0. 02 M buffers at various *p*H values and allowed to stand 2 hr at 30 C. Assays for lytic activity in the supernatant fluids, after removal of cells and debris by centrifugation, showed that enzymes were released

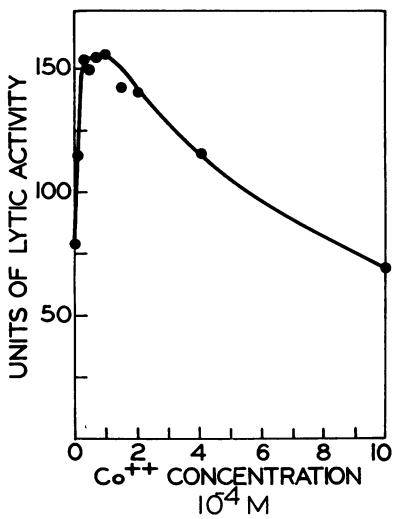

FIG. 2. *Effect of Co^{++} concentration of lysis of Bacillus thuringiensis cell walls by dialyzed crude autolysate at pH 8.5. Reaction mixtures, containing 120 μliters of 0.1 M Tris-chloride buffer, pH 8.5, 50 μliters of a dialyzed autolysate, varying quantities of $CoCl_2$, and water to 400 μliters, were preincubated for 10 min at 37 C. Then 200 μliters (0.6 mg) of a cell wall suspension was added. The optical density at 650 nm was followed during incubation at 37 C for 10 min. One unit of activity is defined as a change in optical density of 0.001 in 5 min. The zero point on the abscissa refers to activity in the absence of added Co^{++}.*

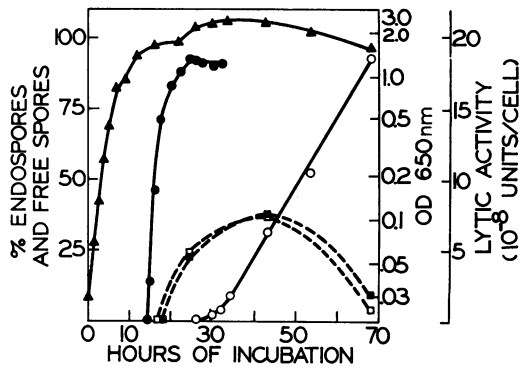

FIG. 3. *Lytic activity in extracts obtained from Bacillus thuringiensis cells at various times during growth and sporulation. Optical density at 650 nm and the ratio of phase light endospores and free spores to the total number of cells were determined. At various times, samples containing 150 to 250 mg of cells (dry weight) were removed. The cells were sedimented by centrifugation, resuspended in 10 ml of 0.02 M potassium phosphate buffer, pH 7.8, and the vegetative cells and sporangia were then disrupted by sonic oscillation. After centrifugation to remove residual intact cells, spores and debris, the supernatant fluids were assayed for lytic activity on purified cell walls at pH 4.1 and pH 8.5. Symbols: ▲, optical density; ●, endospores (% of total cells); ○, free spores (% of total cells); ■, pH 4.1 lytic activity in cell extracts; □, pH 8.5 lytic activity in cell extracts.*

over a broad *p*H range. The maximal amount of activity was released near *p*H 8. All subsequent autolysates were prepared by suspending cells in 0.02 M potassium phosphate buffer, *p*H 7.8.

Several membrane-solubilizing agents were tested for their effect on the release of lytic enzymes. Cells were allowed to autolyze in the presence of sodium dodecyl sulfate, sodium deoxycholate, and toluene for 2 hr. Lytic activity and protein content of the supernatant fluids were determined after centrifugation to remove cells and debris (Table 2). Autolysis in the presence of the surface active agents resulted in the release of 50 to 60% more *p*H 4.1 lytic activity and 200 to 300% more *p*H 8.5 activity than was obtained by autolysis in buffer. The agents also released a large amount of protein. Of the three agents tested, toluene proved to be superior and was used in the preparation of autolysates for purification of the lytic enzymes.

Isolation and purification of the lytic enzymes. All purification procedures were carried out at 4 C. Some preliminary experiments revealed that the *p*H 4.1 and 8.5 activities were both precipitated between 30 and 60% $(NH_4)_2SO_4$ saturation and between 0.5 and 1.5 volumes of cold acetone. Strange and Dark (11) reported that acidification of a crude autolysate of sporulating *B. cereus* to *p*H 3.0 resulted in the precipitation of most of the enzyme activity active at *p*H 8.5 while leaving the *p*H 4.1 activity in solution. Because of the apparent similarity of the *B. thuringiensis* auto-

TABLE 2. *Effect of toluene, sodium dodecyl sulfate (SDS), and sodium deoxycholate (SDC) on the release of lytic activity from sporulating cells of Bacillus thuringiensis*

Addition to autolyzing system	Lytic activity			
	*p*H 4.1		*p*H 8.5	
	Units/ml[a]	Specific activity	Units /ml[a]	Specific activity
None	780	813	540	573
Toluene, 2.5% (v/v)	1,260	566	1,760	789
SDS, 0.33% (w/v)	1,040	331	1,380	440
SDC, 0.33% (w/v)	1,200	426	1,640	582

[a] One unit of lytic activity is defined as that amount of enzyme giving an initial linear decrease in optical density of 0.001 in 5 min at 650 nm.

lytic system, the effect of lowering the *p*H was tested. Approximately 60% of the *p*H 8.5 activity was precipitated when the crude autolysate was adjusted to *p*H 4.0 by the addition of HCl. There was no increase in the precipitation of protein or lytic activity upon lowering the *p*H further. The *p*H 4.1 activity and 40% of the *p*H 8.5 activity remained soluble. Because of the incomplete separation of the two activities, this procedure was not utilized in further attempts at separation and purification.

A two-step procedure resulted in a separation of the *p*H 4.1 and 8.5 activities (Table 3). The autolysate from 89 g (wet weight) of cells was adjusted to *p*H 7.5. Sufficient 1 M potassium phosphate buffer, *p*H 7.5, was then added to bring the solution to 0.1 M. The required amount of solid $(NH_4)_2SO_4$ to bring the solution to 30% saturation was added slowly with stirring. A small amount of an inactive precipitate formed during stirring for an additional 60 min and was removed by vacuum filtration through a Celite pad on a Büchner funnel. The pad was rinsed with 100 ml of a cold solution of buffer saturated to 30% with $(NH_4)_2SO_4$. The combined filtrate and wash were then brought to 65% $(NH_4)_2SO_4$ saturation by the slow addition of the solid salt. After 4 hr of slow stirring in an ice bath, the precipitate was sedimented by centrifugation at 57,000 × *g* for 30 min. The supernatant fluid, which contained no lytic activity at *p*H 4.1 or *p*H 8.5, was discarded. The precipitate was dissolved in 32 ml of 0.05 M Tris-chloride buffer, *p*H 7.8, and dialyzed against 9 liters of 0.01 M Tris-chloride buffer, *p*H 7.8, for 10 hr and then against 9 liters of fresh buffer for 5 hr. A slight precipitate which contained no activity formed during dialysis and was removed by centrifugation. The net result of this procedure was a dark brown, slightly viscous solution containing both the *p*H 4.1 and 8.5 activities. The *p*H 4.1 activity was purified nearly twofold with a recovery of 71% of the original activity, whereas the *p*H 8.5 activity was purified 2.3-fold with 84% recovery.

TABLE 3. *Initial separation and purification of lytic enzymes*[a]

Fraction	Units/ml	Total units[b] (10^6)	Recovery (%)	Specific activity
Crude autolysate	1,320	10.8	100	562
	1,500	12.3	100	638
$(NH_4)_2SO_4$, 30 to 65%	17,000	7.66	70.8	1,120
	22,800	10.3	83.5	1,500
DEAE-cellulose (first peak)	1,100	1.11	10.3	1,450
	5,200	5.28	42.9	6,890
DEAE-cellulose (second peak)	3,230	6.59	61.0	3,890
	150	0.31	2.5	180

[a] The top figure in each instance corresponds to lytic activity when assayed at *p*H 4.1; the bottom figure corresponds to activity assayed at *p*H 8.5.

[b] Total units are corrected for samples retained at each step for activity and protein determinations.

The dialyzed solution of the $(NH_4)_2SO_4$ precipitate was applied to a 1.7- by 24-cm column of DEAE-cellulose. Fractions of 10 ml were collected. The column was first washed with 300 ml of 0.01 M Tris-chloride buffer, *p*H 7.8, followed with 250 ml of 0.05 M NaCl, then with 500 ml of 0.2 M NaCl, and finally with 150 ml of 1.0 M NaCl. In each case, the salt was dissolved in the buffer. The results of this elution procedure are shown in Fig. 4. Fractions 5 through 13 (first peak) contained the bulk of the *p*H 8.5 activity. Analyses of the pooled fractions showed a total purification of this activity of nearly 12-fold, with an overall recovery of 43%. This peak also contained 10% of the *p*H 4.1 activity, but its specific activity was essentially the same as for the prior ammonium sulfate step. Fractions 65 through 79 (second peak) contained almost exclusively the *p*H 4.1 activity. The pooled fractions showed a total purification of 6.9-fold, with an overall recovery of 61%. It is not known whether the small amount of *p*H 4.1 activity in the first peak and *p*H 8.5 activity in the second peak represents incomplete separation of the two activities or, alternatively, the slight amount of activity which one might expect at the "tailing" of the *p*H optimal curve for each enzyme.

The lytic enzyme with the *p*H optimum at 4.1 was purified further (Table 4). The pooled fractions from peak 2 of the DEAE fractionation above were lyophilized to dryness, dissolved in 9.0 ml of cold distilled water, and 3.0 ml was applied to a 2.6- by 60-cm column of Sephadex G-200. Phosphate buffer (0.02 M, *p*H 6.5) was passed through the column, and 10-ml fractions were collected. A single peak of lytic activity was obtained (Fig. 5). The pooled active fractions (12 through 30) showed an overall purification of 8.9-fold, with a recovery of 55%. The enzyme solution was adjusted to *p*H 4.0 by the addition of 1 N HCl. A slight precipitate, containing no lytic activity, was removed by centrifugation at 15,000 × *g* for 60 min. This procedure gave only a small increase in purification. The supernatant fluid was lyophilized, dissolved in 42 ml of cold water, and then dialyzed twice against 4 liters of 0.01 M Tris-chloride buffer, *p*H 7.3, for 8 hr. A

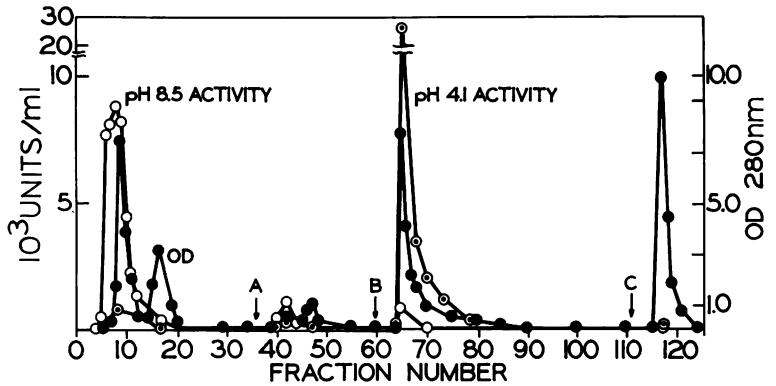

FIG. 4. *Separation and purification of lytic activities by DEAE-cellulose column chromatography. To a 1.7- by 24-cm column of DEAE-cellulose was added 44 ml of dialyzed enzyme solution obtained from ammonium sulfate fractionation. The column was washed with 0.01 M Tris-chloride buffer, pH 7.8, and 10-ml fractions were collected. NaCl was added to the elution buffer in the final concentration of 0.05 M at point A, 0.2 M at point B, and 1.0 M at point C. Fractions were analyzed for protein content (optical density at 280 nm) and lytic activity at pH 4.1 and pH 8.5. Symbols: ●, optical density at 280 nm: ○, pH 8.5 lytic activity: ◉, pH 4.1 lytic activity.*

TABLE 4. *Further purification of the pH 4.1 lytic enzyme*

Fraction	Units/ml	Total units[a] (10^5)	Recovery[b] (%)	Specific activity
Sephadex G-200 column[c]	960	5.89	54.5	5,000
Supernatant fluid, *p*H 4.0	930	5.73	53.1	5,280
Dialysis, *p*H 7.3	4,000	4.96	45.9	14,200

[a] Total units are corrected for samples retained at each step for activity and protein determinations.

[b] Recoveries are relative to the crude autolysate (*see* Table 3).

[c] One-third of the material from the DEAE-cellulose step (*see* Table 3) was applied to the G-200 column. All values for total units and recovery in this table have, therefore, been multiplied by a factor of 3.

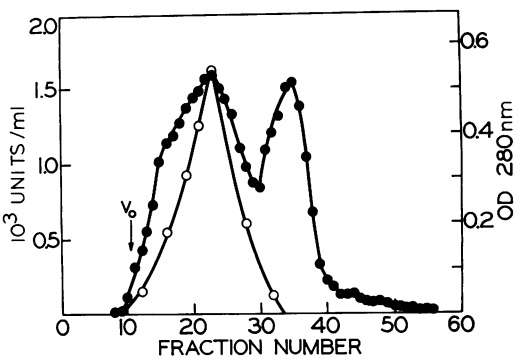

FIG. 5. *Purification of the pH 4.1 enzyme by gel filtration. To a 2.6- by 60-cm column of Sephadex G-200 was applied 3 ml of the concentrated enzyme obtained from the second peak of the DEAE-cellulose column (Fig. 4). The column was washed with 0.02 M potassium phosphate buffer, pH 6.5, and 10-ml fractions were collected. Fractions were analyzed for protein content (optical density at 280 nm) and lytic activity at pH 4.1. Symbols: ●, optical density at 280 nm; ○, lytic activity; V_0, void volume of the column.*

large amount of a flocculent, enzymatically inactive precipitate formed during dialysis and was removed by centrifugation. The dialysis and removal of precipitate resulted in more than a doubling of the specific activity of the supernatant fluid. As compared to the starting autolysate, the *p*H 4.1 activity had been purified 25.3-fold with a recovery of 46%.

The pooled fractions from the first peak of the DEAE column (Fig. 4), which contained the *p*H 8.5 activity, were lyophilized to dryness and then dissolved in 4.0 ml of water. The concentrated enzyme solution was applied to a 1.7- by 45-cm column of DEAE-cellulose over which was layered 6 cm of Sephadex G-25. The purpose of the Sephadex layer was to desalt the sample before it entered the DEAE-cellulose. The enzyme was eluted from the column with 0.01 M Tris-chloride buffer, *p*H 7.8. The enzymatic activity was not adsorbed to the column but was eluted as a sharp band followed by a broad peak of inactive protein. The pooled active fractions showed an overall increase in specific activity of 30.8-fold, with a recovery of 32% (Table 5). The pooled fractions were lyophilized, dissolved in 3.0 ml of water, and applied to a 1.8- by 53-cm column of Sephadex G-100. Tris-chloride buffer (0.01 M, *p*H 7.8) was

passed through the column, and 5.0 ml fractions were collected. Two distinct peaks of activity were obtained (Fig. 6). The first peak, containing most of the activity, will be referred to as peak A and the second as peak B. The fractions comprising the two peaks were pooled separately and lyophilized. Each was then dissolved in 8.0 ml of water and dialyzed twice for 8 hr against 4 liters of 0.01 M Tris-chloride buffer, *p*H 7.3. A slight precipitate, containing no lytic activity, formed during dialysis of the material from both peaks and was removed by centrifugation. With respect to the initial *p*H 8.5 activity of the crude autolysate, the final purification of the peak A activity was 39.8-fold, and the peak B activity was 30.6-fold. The final recovery of *p*H 8.5 activity, calculated from the sum of the activities of peaks A and B, was 16%.

A determination was made of the effect on the rate of cell wall lysis of preincubating samples from peaks A and B with 10^{-4} M Co^{++} for 10 min prior to the addition of substrate. The activity of the peak A fraction was stimulated by 160%, whereas the peak B activity was stimulated only 35%. The slight stimulation of the latter probably results from the presence of some of the peak A activity.

Modes of lytic activity of the purified enzymes. The release of reducing power, free amino acids, and N- and C-terminal amino groups was followed during digestion of cell walls of *B. thuringiensis* by each of the enzymes. Figure 7A shows that the peak A fraction of *p*H 8.5 activity released only N-terminal alanine groups from the cell walls. A maximum of 55 to 60% of the theoretical number of susceptible bonds were hydrolyzed. No reducing power was released during the digestion. It can be concluded that cell wall degradation by this fraction is due to the action of an *N*-acetylmuramyl-L-alanine amidase.

TABLE 5. *Further purification of pH 8.5 lytic enzymes*

Fraction	Units/ml	Total units[a] (10^6)	Recovery[b] (%)	Specific activity
Second DEAE-cellulose column	10,300	3.87	31.5	19,600
Sephadex G-100 column[c]	2,350	1.88	19.2[d]	17,800
	1,020	0.47		7,180
Dialysis,[c] *p*H 7.3	12,800	1.60	15.6[d]	25,400
	2,500	0.32		19,500

[a] Total units are corrected for samples retained at each step for activity and protein determinations.

[b] Recoveries are relative to the crude autolysate (*see* Table 3).

[c] The top figure in each case corresponds to the peak A fraction from the Sephadex G-100 column and the bottom figure to the peak B fraction.

[d] These values are based upon the sum of the total units of both peaks of activity.

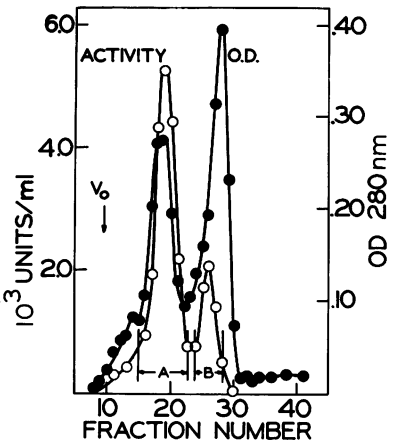

FIG. 6. *Separation of pH 8.5 enzymes by gel filtration. To a 1.8- by 53-cm column of Sephadex G-100 was applied 2 ml of the concentrated enzyme obtained from the second DEAE-cellulose column. The column was washed with 0.01 M Tris-chloride buffer, pH 7.8, and 5.0-ml fractions were collected. Fractions were analyzed for protein content (optical density at 280 nm) and lytic activity at pH 8.5. Symbols: ●, optical density at 280 nm; ○, lytic activity, V_0, void volume of the column.*

Lysis of cell walls by the peak B fraction of the *p*H 8.5 activity was accompanied by the release of N-terminal groups of alanine and glutamic acid and C-terminal groups of alanine (Fig. 7B). The liberation of N-terminal groups of alanine in the absence of any increase in C-terminal diaminopimelic acid indicates the action of an *N*-acetylmuramyl-L-alanine amidase. The liberation of N-terminal glutamic acid accompanied by an increase in C-terminal alanine is evidence for the activity of an endopeptidase which splits the peptide bond between L-alanine and D-glutamic acid. There was no release of reducing power or of other N- and C-terminal amino acids. It is not known whether the amidase and peptidase activities are catalyzed by a single enzyme or a combination of two enzymes. The fact that the peak A activity released no N-terminal glutamic acid (Fig. 7A) indicates that this fraction is free of the endopeptidase.

The purified *p*H 4.1 enzyme released only reducing power during digestion of the cell walls (Fig. 7C), indicating that this enzyme is a glycosidase. The nature of the susceptible glycosidic bond was

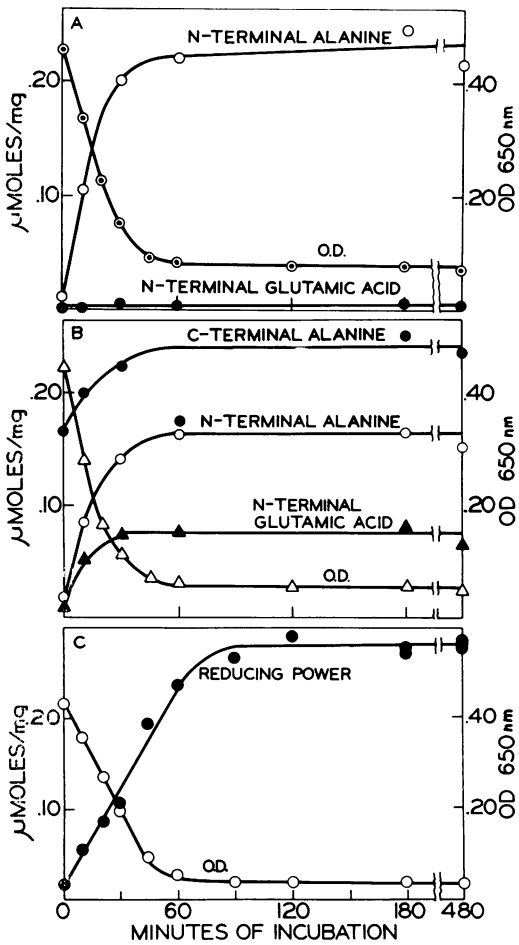

FIG. 7. *Release of N- and C-terminal amino acid groups and reducing power from cell walls of Bacillus thuringiensis by three purified autolytic enzymes. Part A: pH 8.5 peak A enzyme. A 4.0-ml reaction mixture containing 50 μliters of purified peak A enzyme, 0.03 M Tris-chloride buffer, pH 8.5, 1.5 × 10^{-4} M $CoCl_2$, and 50 μliters of 1% mercaptoethanol was preincubated for 10 min at 37 C. After addition of 2.0 ml of cell wall suspension (6.0 mg), incubation was continued. At various times, optical density was determined, and samples were removed for the determination of reducing power and free and N- and C-terminal amino acids. Part B: pH 8.5 peak B enzyme. The procedure was identical to that outlined above, except that 250 μliters of the peak B enzyme was used. Part C: pH 4.1 enzyme. The reaction mixture contained, in a total volume of 4.0 ml, 100 μliters of enzyme and 0.03 M potassium phthalate-NaOH buffer, pH 4.1. After incubation for 10 min at 37 C, 2 ml (6.0 mg) of cell wall suspension was added. The remainder of the procedure was identical to that described above.*

determined after reduction of a cell wall digest (3). Samples of digested and undigested cell walls were reduced with 0.1 M sodium borohydride for 3 hr at room temperature. The excess reagent was destroyed with acetic acid, and the samples were dried, acid-hydrolyzed, and analyzed using the amino acid analyzer. Muramitol (produced by reduction of free reducing-end groups of muramic acid) is eluted in the same position as muramic acid but gives almost no color with ninhydrin. Glucosaminitol (the reduced product of free glucosamine) is eluted in a position different from that of glucosamine. Reduction of the enzymatic digest resulted in a decrease in muramic acid of 58%, as compared to that in undigested walls. There was no decrease in glucosamine content. This indicates that the *p*H 4.1 enzyme is a *N*-acetylmuramidase, an enzyme which cleaves the glycosidic bond linking the 4-position of *N*-acetylglucosamine to the 1-position (reducing end) of *N*-acetyl muramic acid.

The crude autolysate was assayed for its ability to release reducing power, N- and C-terminal amino acid groups, and free amino acids during cell wall digestion. At *p*H 8.5, both amidase and endopeptidase activities were present, and there was no glycosidase activity. At *p*H 4.1, glycosidase activity was observed, and there was only a slight trace of amidase activity.

Survey of the lytic activity of the purified enzymes against cell walls of various bacteria. The susceptibility of a variety of bacterial cell walls to each of the lytic enzymes was tested. The enzymes exhibited a surprisingly limited range of activity. With the exception of *M. lysodeikticus* cell walls, which were highly susceptible to the muramidase, the three enzymes attacked only cell walls of the genus *Bacillus* (Table 6). The muramidase and

TABLE 6. *Survey of the activity of the three lytic fractions against cell walls of various bacteria*

Cell walls from	Rate of decrease in optical density at 650 nm		
	*p*H 4 muramidase (5 min)	*p*H 8.5 amidase (5 min)	*p*H 8.5 peptidase (10 min)
Bacillus thuringiensis var. *thuringiensis*	.20	.17	.22
B. megaterium	.23	.15	.06
B. cereus T	.25	.17	.08
B. subtilis Marburg	.37	.01	.09
B. subtilis Porton	.34	.01	.07
B. polymyxa	.16	.00	.06
Bacillus sp.	.21	.02	.20
M. lysodeikticus	.30	.00	.00

peptidase exhibit somewhat broader specificity than the amidase, the latter attacking only three of the seven preparations of cell walls of this genus. Cell walls of the following bacteria proved completely resistant to the three enzymes: *M. roseus*, *Staphylococcus aureus*, *S. epidermidis*, *Clostridium histolyticum*, *Lactobacillus casei*, *Arthrobacter crystallopoietes*, *Spirillum serpens* (peptidoglycan layer), and *Azotobacter vinelandii*.

DISCUSSION

The results of this investigation indicate that autolysis of sporulating cells of *B. thuringiensis* is brought about by the action of three distinct cell wall lytic enzymes. A model of the *B. thuringiensis* peptidoglycan showing the sites of attack of these three enzymes is presented in Fig. 8. The construction of this model is based upon our previously published description of the chemical structure of this peptidoglycan (6). The glycan constituent of the polymer is hydrolyzed by an *N*-acetylmuramidase. This type of activity seems to be the most ubiquitous among the bacteriolytic enzymes. Examples are the lysozyme found in plants, egg white, and animal tissues, the F_1 and 32 enzymes of *Streptomyces albus* G, the B enzyme of *Chalaropsis*, and the T_2 phage lytic enzyme [(*see* Table 1 of (3)] for specific references to each of the above). The *B. thuringiensis* muramidase differs from most of the above in its low *p*H optimum of 4.0 and the extremely limited range of cell walls which can serve as substrate.

The peptide moiety of the peptidoglycan is attacked by the two enzymes with the *p*H optima at 8.5. The enzyme clearly stimulated by cobalt ions is an *N*-acetylmuramyl-L-alanine amidase. This type of activity is fairly common among the bacteriolytic enzymes produced by microorganisms (3, Table 1). The second enzyme, apparently unaffected by cobalt ions, is an L-alanine-D-glutamic

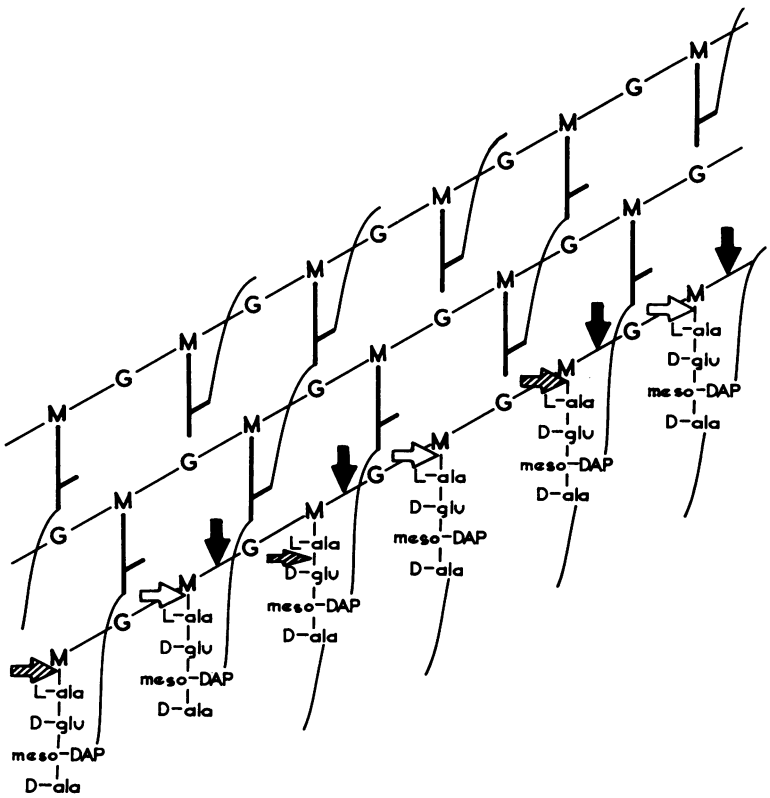

FIG. 8. *Hypothetical structural model of the peptidoglycan component of Bacillus thuringiensis cell walls showing the sites of activity of three autolytic enzymes. M and G represent, respectively, N-acetylmuramic acid and N-acetylglucosamine. The heavy lines attached to M represent the tetrapeptide. The curved lines represent the direct tetrapeptide cross-bridge peptide bonds. The model is based upon previously published analyses of this peptidoglycan (7). Symbols: open arrow, pH 8.5, peak A N-acetylmuramyl-L-alanine amidase; shaded arrow, pH 8.5, peak B amidase and endopeptidase; solid arrow, pH 4.1 N-acetylmuramidase.*

acid endopeptidase. No endopeptidase with this particular specificity has been previously described. This enzyme will be a valuable addition to the stockpile of cell wall lytic enzymes available for studies of cell wall chemistry, especially so if completely freed from the amidase activity.

Strange and Dark (11) observed two cell wall lytic enzymes in sporulating cells of *B. cereus*. One had a *p*H optimum of 8.0, was stimulated by cobalt ions, and was precipitated at *p*H 3.0. The other was most active at *p*H 4.5 and was soluble at *p*H 3.0. The former enzyme, but not the latter, was detected in spores of the organism (10). There are striking similarities between the lytic enzymes of *B. cereus* and those produced during sporulation of *B. thuringiensis*. This correlates with the reports that these two organisms are closely related taxonomically (5, 7). We did not examine extracts of spores to determine whether the cobalt-stimulated amidase was present. A final decision as to the relatedness of the autolytic enzymes of *B. cereus* and *B. thuringiensis* must await determination of the modes of lytic activity by the former.

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