Metalloprotease from *Bacillus thuringiensis*

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*Bacillus thuringiensis* var. *kurstaki* was shown to produce an extracellular, metal chelator-sensitive protease during the early stages of sporulation. Protease production in nutrient broth was dependent upon supplementation with Mn$^{2+}$ or Ca$^{2+}$. The addition of Ca$^{2+}$ was required for enzyme stabilization. Protease production occurred in nutrient broth supplemented with $7 \times 10^{-3}$ M Ca$^{2+}$, $5 \times 10^{-4}$ M Mn$^{2+}$, and $10^{-3}$ M Mg$^{2+}$. The protease had optimum activity in the pH range 6.5 to 7.5. It was inhibited by chelating agents but not by a serine protease inhibitor. The culture supernatant and the partially purified protease lacked esterase activity. Partial purification of the enzyme (92.3-fold) by (NH$_4$)$_2$SO$_4$ fractionation and starch adsorption yielded an enzyme whose molecular weight was estimated to be 37,500 by acrylamide gel-sodium dodecyl sulfate electrophoresis or 40,800 by sucrose density gradient centrifugation. In the presence of Ca$^{2+}$, the partially purified enzyme retained 78% of its activity after heating at 70 C for 10 min but only 8% of its activity after heating at 80 C for 10 min.

The nature of proteolytic enzymes of microbial origin is less well known than that of the proteolytic enzymes of animal or plant origin. In recent years, however, the discovery of applied uses for some of the microbial enzymes and their possible relationships to sporulation in bacilli (16) has served to stimulate research in this area. Morihara (12) has classified the microbial proteases into four groups. These are (i) the serine proteases formed by some Streptomyces and by *Bacillus subtilis* (the subtilisins), (ii) the thiol proteases formed by Clostridium histolyticum and Group A streptococci, (iii) the acid proteases formed by aspergilli and some other fungi, and (iv) the metal chelator-sensitive proteases formed by *B. subtilis, Bacillus megaterium, Bacillus thermoproteolyticus, Bacillus polymyxa*, and *Bacillus cereus* (4, 6, 12). The ability to synthesize serine and/or metal chelator-sensitive protease seems to be widespread among bacteria of the genus *Bacillus*. Whether or not these enzymes have a specific role in sporulation or some other aspect of secondary metabolism is an open question.

One member of the genus, *Bacillus thuringiensis*, forms a proteinaceous parasporal or crystal at the time of sporulation. The parasporal is responsible for the toxicity of the microorganism for *Lepidoptera* larvae (15). With the exception of a report dealing with enzymes associated with mature endospore release (9), no information is available about the extracellular hydrolytic enzymes produced by *B. thuringiensis* or about the possible relationship of extracellular protease to parasporal formation. In this report we present information about the first part of this question, i.e., the nature of extracellular protease produced by *B. thuringiensis*. We describe the appearance early in the sporulation sequence of an extracellular, metal chelator-sensitive protease, the conditions for its synthesis, and some of its properties.

**MATERIALS AND METHODS**

Organism and cultural conditions. *B. thuringiensis* var. *kurstaki* (strain HD-1) was maintained on slants of nutrient sporulation agar (NSM). NSM agar was slightly modified from the formulation of Fortnagel and Freese (5) and contained 23 g of nutrient agar (Difco) per liter, $7 \times 10^{-3}$ M CaCl$_2$, $5 \times 10^{-4}$ M MnCl$_2$, and $10^{-3}$ M MgCl$_2$. Inocula for growth curve experiments were prepared by a modification of the active culture technique of Nakata and Halvorson (13). This consisted of overnight static growth in nutrient broth (Difco) at 30 C, followed by the inoculation of 10 ml of nutrient broth in a 125-ml flask with 1.0 ml from the static flask. The 10 ml of broth were shaken (200 rpm, New Brunswick G76 shaker) for 3 h at 32 C, and 1.0 ml was then used to inoculate a second 10 ml of nutrient broth. This transfer was repeated with a third shaken flask and this was used as the source of young cells to make a 10% (vol/vol) inoculation of 50 ml of broth in a 500-ml flask for the growth curves. The 50 ml of broth for all growth curves were shaken at 220 rpm (32 C). Growth was followed by measuring absorbance at 660 nm in a Klett-Summerson colorimeter with 12.5-mm cuvettes.
Formation of heat-stable spores was determined by removing 1.0 ml of broth from a growth curve flask, sonicating the 1.0 ml for 1 min to deagulum cells, heating at 80°C for 12 min, diluting in water, and plating in nutrient agar. Declumping was carried out by placing 1.0 ml of cells and spores in a sterile plastic tube (12 by 75 mm), floating the tube in water in the chamber of a Ratheon 10-ke sonic oscillator, and treating at full power for 1 min.

Culture supernatant from which the B. thuringiensis protease was purified was prepared by inoculating 200 ml of protease production medium in a 2-liter flask with 20 ml of B. thuringiensis from the last flask in an active culture sequence. Protease production medium contained 8 g of nutrient broth per liter, 7 × 10⁻³ M CaCl₂, 5 × 10⁻⁴ M MnCl₂, and 10⁻³ M MgCl₂. The 2-liter flasks containing protease production medium were shaken (220 rpm, New Brunswick G25 shaker) for 12 h at 32°C and the cells were removed by centrifugation.

Enzyme assays. Protease activity was determined by digestion of azo-albumin. Each reaction mixture was run in duplicate and contained 1.0 ml of 0.5% (wt/vol) azo-albumin which had been prepared in 0.1 M buffer at the appropriate pH (see below), 0.7 ml of water, and 0.3 ml of enzyme preparation. The mixture was incubated 1 h at 30°C, the reaction was stopped by addition of 2.0 ml of 8% trichloroacetic acid, and the precipitate was removed by centrifugation (27,000 × g for 15 min). Two milliliters of the supernatant were added to 2.0 ml of 0.5 M NaOH and the absorbance was measured in a 1.0-cm cuvette at 440 nm in a Hitachi Perkin-Elmer 139 spectrophotometer. The blank was prepared by adding trichloroacetic acid to the substrate before adding enzyme. One unit of protease is that amount of enzyme which produced an absorbance increase of 0.01 per h under the assay conditions. Specific activity is units of protease per milligram of protein. When enzyme activity at various pH's was determined, substrate was prepared in the following 0.1 M buffers: pH 5.8 and 6.1 in 2(N-morpholino) ethanol sulfonic acid; pH 6.5, 7.0, 7.5, and 7.9 in morpholinopropane sulfonic acid (MOPS); pH 8.5 in tri(hydroxymethyl)methylaminomethane; and pH 9.10 and 9.75 in glycin NaOH.

Amylase was determined by the method of Bernfield (1) using 1% soluble starch (Difco) in 0.02 M potassium phosphate buffer, pH 6.9, as substrate. One unit of amylase liberated 1.0 mg of maltose in 3 min at 25°C.

Esterase was determined by the method of Prestidge et al. (14) using benzoylarginine ethyl ester, acetyltyrosine ethyl ester, or p-nitrophenylacetate as substrate.

Protein was measured by the method of Lowry et al. (10) using bovine serum albumin as a standard.

Enzyme purification. Crude culture supernatant prepared as previously described was concentrated 18.7-fold by ultrafiltration with an Amicon stirred cell (model 52) using a Diaflo ultrafilter UM10 membrane under 5 lb/in² of nitrogen at 5°C. The concentrated culture supernatant was clarified by centrifugation at 27,000 × g for 20 min and 2.0% (wt/vol) calcium acetate was added. Solid (NH₄)₂SO₄ was added slowly, with constant stirring to the concentration, and the mixture was allowed to stand 10 min at 4°C before centrifugal recovery of the precipitate at 45, 50, and 65% saturation. The precipitates were dissolved in 0.1 M MOPS-0.1% calcium acetate buffer, pH 7.0, and dialyzed against the same buffer at 4°C for 6 h. Amylase and pigments were removed by adding 10% (wt/vol) pulverized potato starch and 12% (vol/vol) ethanol to the dialyzed (NH₄)₂SO₄ fraction. The enzyme preparation was stirred for 10 min at 4°C and the starch was removed by vacuum filtration. The procedure was repeated twice.

Acrylamide gel disc electrophoresis. The number of proteins present at various stages of purification and the apparent molecular weight of the 92.3 × 10⁻³ pure protease were determined (17) by electrophoresis at 24°C in tubes of 7.5% acrylamide-0.1% sodium dodecyl sulfate at pH 7.2 (0.1 M sodium phosphate buffer). Electrophoresis was performed from cathode to anode in a unit supplied by Hoefer Scientific Instruments (San Francisco, Calif.) with a constant current of 4 mA per tube. Samples (250 μl) were prepared by adding 0.1 volume of 10% sodium dodecyl sulfate in 0.01 M sodium phosphate buffer, pH 7.2, and 0.1 volume of 10% mercaptoethanol to the enzyme. The mixture was boiled 1 min and cooled. Two drops of glycerol and two drops of dilute bromophenol blue were added and the sample was applied to the top of the gel. After electrophoresis for 10 to 12 h, the gels were removed, fixed in 20% sulfosalicylic acid for 8 to 12 h, stained for 14 h with 0.25% Coomassie brilliant blue, and destained with 7.0% acetic acid in a diffusion destainer (Hoefer Scientific Instruments, Inc.). For molecular weight determinations the gels were scanned at 540 nm in a Gilford spectrophotometer equipped with linear transport. The distance from the top of the gel to the peak of absorbance indicated on the paper was measured and plotted against the molecular weight of the protein to produce a standard curve. Standard proteins used and their molecular weights were bovine hemoglobin (16,000), pepsin (35,000), ovalbumin (43,500), and bovine serum albumin (65,400).

The single protein band found upon electrophoresis of the 92.3 × 10⁻³ purified protease was proven to be protease by preparing and electrophoresing the sample under nondenaturing conditions. The gel was cut into 5-mm segments, each segment was crushed with a glass rod in the bottom of a culture tube, and 1.0 ml of 0.01 M MOPS-0.1% calcium acetate (pH 7.2) and 1.0 ml of 5 mg of azo-albumin per ml were added. The segments were incubated 24 h at 30°C and the protease activity was determined as previously described.

Sucrose density gradient centrifugation. The molecular weight of the 92.3 × 10⁻³ purified protease was determined by centrifugation in the SW50.1 head of a Beckman model L3-50 ultracentrifuge using a 5 to 20% sucrose gradient. Calculation of the molecular weight and assumptions regarding the partial specific volume of the protease are described by Martin and Ames (11). The standard protein was bovine hemoglobin. Five-drop samples were collected by bottom puncture of the centrifuge tube. Hemoglobin was located by absorbance at 405 nm and protease by assay of each fraction.

Chemicals. Azo-albumin, benzoylarginine ethyl...
ester, acetyltyrosine ethyl ester, p-nitrophenylacetate, bovine hemoglobin, bovine serum albumin, ovalbumin, pepsin, o-phenanthroline, ethylenediaminetetraacetic acid, tris(hydroxymethyl)aminomethane, (N-morpholino)ethane sulfonic acid, sodium dodecyl sulfate, Coomassie brilliant blue R, and phenylmethyl sulfonyl fluoride were obtained from Sigma Chemical Co., St. Louis, Mo.

Acrylamide, bis-acrylamide, 2-mercaptoethanol, ammonium persulfate, and N,N,N',N'-tetramethyl-ethylenediamine were obtained from Eastman Kodak Co., Rochester, N. Y.

Maltose and soluble starch were obtained from Difco Laboratories, Detroit, Mich.

Pulverized potato starch was obtained from Baker and Adamson Chemicals, Morristown, N. J.

RESULTS

Protease response to pH and inhibitors. B. thuringiensis was grown 12 h in NSM broth and the protease activity in the culture supernatant was assayed at several different pH's. Figure 1 illustrates the activity response to pH. The activity was maximum in the pH 6.5 to 7.5 range and declined rapidly below pH 6.5. The activity was maintained at a higher level in the alkaline range although it had declined markedly by pH 9.1. The loss of activity in the alkaline pH range suggested that most if not all of the activity was due to a metal chelator-sensitive protease (sometimes referred to as a neutral protease). This was tested by the addition of 1.0 mM o-phenanthroline to protease assay mixtures at pH 7.0 and 8.5. The activity was inhibited by 97 and 86% at pH 7.0 and 8.5, respectively (Fig. 1). Ethylenediaminetetraacetic acid (50 mM) inhibited protease activity 89% at pH 7.0 and 1.0 mM phenylmethyl sulfonyl fluoride, a serine protease inhibitor, was not inhibitory at pH 7.0 or 8.5.

Time of appearance of proteolytic activity. Extracellular proteases of bacilli have often been shown to appear at the beginning of the stationary phase of growth (16). Figure 2 reveals that B. thuringiensis produced only 25 U of activity through h 4, the time of completion of exponential growth. From the h 4 to 9, protease activity increased steadily, reaching a maximum of 180 U at h 9. The peak in protease activity occurred within 1 h of the appearance of heat-stable spores. Phase contrast microscopy examination showed little or no cell lysis during the period when protease activity appeared in the medium. Further indication that the protease activity was excreted rather than the result of cell lysis was the absence of esterase activity against p-nitrophenylacetate in the supernatant. Cell extracts made by sonication contained very active esterase activity against this substrate.

Effect of medium on protease production. NSM broth was used for the experiments described above because preliminary experiments had shown it to allow greater protease production than glucose-yeast extract-salts broth (19), casein-casitone-glucose broth (3), or yeast extract broth (3). The role of the metal salts in NSM broth was investigated by growing B. thuringiensis in shake flasks of nutrient broth containing each of the metals, Mn²⁺, Mg²⁺, and Ca²⁺, individually. The data presented in Fig. 3 show that no more than 25 U of activity was produced in flasks containing nutrient broth alone or nutrient broth plus 10⁻³ M Mg²⁺. Although 5 × 10⁻⁶ M Mn²⁺ allowed the greatest production of protease of the single metals, the enzyme activity was lost after h 9. The combination of the three salts (NSM broth) allowed production and maintenance of a high level of activity.

The three metals were then tested in pairs with the results given in Fig. 4. Although both the Mn²⁺-Mg²⁺ and the Mn²⁺-Ca²⁺ combinations allowed production of 110 to 120 U of activity in 11 h, the activity in the flask lacking Ca²⁺ was lost after 11 h. The Mg²⁺-Ca²⁺ combination did not support the development of good
activity. The addition of Mg$^{2+}$ to the Mn$^{2+}$-Ca$^{2+}$ combination (NSM broth) allowed production of a high level of activity.

The data in Fig. 3 and 4 demonstrated the importance of certain cations to the development of protease activity in culture supernatants. It was unclear if this was an effect upon enzyme synthesis or if it represented activation of a protein apoenzyme that was excreted into the medium in all of the growth situations tested. If the latter were the case, the supernatant of a nutrient broth culture which had little activity in the usual assay might gain activity if cations were added to the assay tube. This possibility was tested by adding Mg$^{2+}$, Mn$^{2+}$, and Ca$^{2+}$ to a nutrient broth supernatant at a final concentration equal to that in NSM broth and then assaying for protease activity. No increase in activity was observed. The Mg$^{2+}$, Mn$^{2+}$, and Ca$^{2+}$ did not seem to function by activating an apoenzyme.

Several metal chelator-sensitive proteases have been shown to be zinc-containing enzymes (12). The effect on protease production of the addition of various levels of ZnSO$_4$ to NSM broth is presented in Table 1. The addition of as little as $10^{-4}$ M ZnSO$_4$ was inhibitory. Up to $10^{-4}$ M Na$_2$SO$_4$ supplementation of NSM broth had no effect.

**Purification of B. thuringiensis protease.**

The sequence of steps used to achieve partial purification of the *B. thuringiensis* metal chelator-sensitive protease is listed in Table 2. Cells were removed from 2.4 liters of protease production medium broth and the culture supernatant was concentrated 18.7-fold by passage through a UM10 membrane in an Amicon ultrafiltration cell. About 57% of the total activity was retained and the specific activity was increased 1.4×. After the addition of 2% calcium acetate to increase enzyme stability, the concentrated supernatant was fractionated with (NH$_4$)$_2$SO$_4$. Most of the protease activity was found in the 45 to 60% fraction. This step resulted in a total 58.1× increase in specific activity in the 45 to 60% fraction. The amylase activity had also increased (7.2×) in this fraction and brown pigment remained. For the first time more than one band appeared on sodium dodecyl sulfate-acrylamide gels of the enzyme (Fig. 5). Both new bands were slower moving than the main band. Treatment with starch removed all of the brown pigment and 95% of the amylase. Both of the slower moving gel bands were removed by this treatment. The specific activity of the protease had been increased 92.3×. The pH activity profile of the partially purified enzyme was very similar to that found with the crude culture supernatant. The partially purified enzyme was inhibited by ethylenediaminetetraacetic acid and o-phenanthroline to about the same extent as the culture supernatant. The single band found by electrophoresis of the 92.3× purified enzyme was shown to be protease by elution from a nondenaturing gel and activity upon azo-albumin.

**Determination of the apparent molecular weight of the protease.** A semilogarithmic plot of the molecular weight of reference proteins versus band migration distance is shown in Fig. 6. From this plot the apparent molecular weight of the protease was estimated to be 37,
358 Plus protease 10^-5 detected in broth. Enzyme growth. Mn2+, cally in broth supplemented with Mn2+, Mg2+, and Ca2+ excreted a proteolytic enzyme during the early stationary phase of growth. Since the appearance of enzyme activity in the culture supernatant was not accompanied by cell lysis or by the appearance of intra-
cellular esterase activity in the supernatant, the protease was apparently excreted from the intact, sporulating cells. The appearance of extracellular proteolytic activity during the early stages of sporulation resembles the situation reported for B. licheniformis (2) and B. subtilis (14) but is unlike the exponential phase-related synthesis reported in B. polymyxa (6).

The enzyme appeared in the culture supernatant when nutrient broth was supplemented with either Mn2+ or Ca2+ but it appeared at very low levels when nutrient broth was unsup-

![Graph](http://aem.asm.org/Downloaded from http://aem.asm.org/)

**TABLE 1. Effect of Zn2+ addition on protease production in NSM broth**

| Growth (h) | No addition | 10^-4 M Zn2+ | 10^-3 M Zn2+ | 10^-4 M Zn2+ |
|-----------|-------------|-------------|-------------|------------|
| 3         | 35          | 35          | 39          | 33         |
| 6         | 123         | 106         | 43          | 41         |
| 9         | 215         | 155         | 106         | 128        |
| 10        | 212         | 208         | 136         | 93         |
| 12        | 250         | 192         | 175         | 126        |
Table 2. Purification of B. thuringiensis protease

| Steps                        | Vol (ml) | Protein (mg/ml) | Total protein (mg) | Activity (U/ml) | Total activity (U) | Sp act (U/mg) | Purification (-fold) | Enzyme yield | Activity (U/ml) | Sp act (U/mg) |
|------------------------------|----------|-----------------|--------------------|-----------------|--------------------|---------------|---------------------|--------------|-----------------|---------------|
| Crude enzyme solution        | 2,400    | 1.45            | 3,480              | 212             | 508,800            | 146.2         | 1                   | 100          | 0.6             | 0.4           |
| Ultrafiltration cell         | 128      | 11.25           | 1,440              | 2,283           | 292,224            | 202.9         | 1.4                 | 57.4         | 5.9             | 0.5           |
| Ammonium sulfate fractions   |          |                 |                    |                 |                    |               |                     |              |                 |               |
| 0–45%                        | 8        | 0.5             | 4                  | 540             | 4,320              | 1,080         | 7.4                 | 0.8          | 12.6            | 7.2           |
| 45–60%                       | 20       | 1.75            | 35                 | 14,833          | 296,660            | 8,476         | 58.1                | 58.3         | 12.6            | 7.2           |
| 60–65%                       | 8        | 0.89            | 7.1                | 3,200           | 25,600             | 3,605.6       | 24.5                | 5.0          | 1.5             | 1.6           |
| Dialyzed ammonium sulfate    | 17       | 1.6             | 27.2               | 16,333          | 277,661            | 10,208.1      | 69.8                | 54.6         | 12.6            | 7.3           |
| fraction (45–60%)             |          |                 |                    |                 |                    |               |                     |              |                 |               |
| Starch-treated enzyme filtrate| 13       | 0.87            | 11.3               | 11,733          | 152,529            | 13,498.1      | 92.3                | 29.9         | 0.3             | 0.4           |

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of the B. thuringiensis protease at various stages of purification. 1, Crude culture supernatant; 2, concentrated crude culture supernatant; 3, 45 to 60% (NH$_4$)$_2$SO$_4$ fraction; 4, starch-treated enzyme.

Fig. 6. Plot of the molecular weight of reference proteins versus migration distance in sodium dodecyl sulfate-acrylamide gel electrophoresis. 1, Bovine serum albumin; 2, ovalbumin; 3, pepsin; 4, hemoglobin; *, partially purified B. thuringiensis protease.

Almost twice as much protease activity appeared in Mn$^{2+}$-supplemented nutrient broth as in Ca$^{2+}$-supplemented broth but the enzyme activity was not stable in the absence of Ca$^{2+}$. The combinations of Mn$^{2+}$-Ca$^{2+}$ or Mn$^{2+}$-Mg$^{2+}$ allowed good enzyme production but again, in the absence of Ca$^{2+}$ supplement, the activity was lost. These results indicate that Mn$^{2+}$ is important to achieving a high level of enzyme
activity and that Ca²⁺ is important to maintaining that activity in the supernatant. Mn²⁺ is known to be important in the synthesis of various secondary metabolites (18) but its site of action is unknown. It did not appear to be involved in activating a protease apoenzyme. Ca²⁺ is known to stabilize some proteolytic enzymes (12) and it seemed to fulfill that role in these experiments. Mg²⁺ did not by itself support good protease development but, when combined with Mn²⁺ and Ca²⁺, it produced a higher activity than the Mn²⁺-Ca²⁺ pair. Although Zn²⁺ is involved in the activity of several metal chelator-sensitive proteases (12), the addition of Zn²⁺ to the medium was inhibitory rather than stimulatory to enzyme activity. Apparently there was an adequate level of Zn²⁺ in the complex medium and excess was inhibitory.

The information obtained in this study points to the synthesis of a single, metal chelator-sensitive protease, although the existence of very small amounts of other protease types cannot be completely ruled out. The pH profile of enzyme activity showed a peak at neutrality with little residual activity in the pH 9.5 to 10.0 range where serine protease is most active (7).

The enzyme activity was largely but not completely inhibited by chelating agents and the serine protease inhibitor, phenylmethyl sulfonyl fluoride, produced no inhibition at pH 7.0 or 8.5. In view of the very broad pH activity range shown for serine proteases from bacilli (7), their presence should have been detectable by phenylmethyl sulfonyl fluoride inhibition even at pH 7.0 or 8.5. Serine proteases have frequently been shown to have esterase activity (12). The absence of esterase activity in the culture supernatant further argues against the presence of serine protease.

Purifications of the protease free of pigment and much of the amylase provided a preparation giving a single band on disc electrophoresis. The apparent molecular weight of 37,500 determined by disc electrophoresis or 40,800 determined by sucrose density gradient centrifugation is close to the values of 35,100 and 40,500 reported by Keay and Wildi (8) for the metal chelator-sensitive proteases of B. subtilis NRRL B3411 and B. subtilis var. amylolaccariticus, respectively. The metal chelator-sensitive proteases appear to be somewhat larger molecules than the serine proteases whose molecular weights fall in the range of 20,000 to 30,000 (7, 12). Although metal chelator-sensitive proteases are generally reported to be less stable than serine proteases, the B. thuringiensis partially purified protease at pH 7.0 in 0.1% calcium acetate retained most of its activity after 10 min at 70 °C.

LITERATURE CITED

1. Bernfield, P. 1955. Amylases a and B, p. 149. In S. G. Colowick and N. O. Kaplan (ed.). Methods in enzymology, vol. 1. Academic Press Inc., New York.

2. Bernlohr, R. 1964. Postlogarithmic phase metabolism of sporulating microorganisms. I. Protease of Bacillus licheniformis. J. Biol. Chem. 239:538–543.

3. Boyer, H., and B. Carlton. 1968. Production of two proteolytic enzymes by a transformable strain of Bacillus subtilis. Arch. Biochem. Biophys. 128:442–455.

4. Feder, J., L. Keay, L. Garrett, N. Cirulis, M. Moseley, and B. Wildi. 1971. Bacillus cereus neutral protease. Biochim. Biophys. Acta 251:74–78.

5. Fortnagel, P., and E. Freese. 1968. Analysis of sporulation mutants. II. Mutants blocked in the citric acid cycle. J. Bacteriol. 95:1431–1435.

6. Griffin, P., and W. Fogarty. 1973. Physicochemical properties of the native, zinc and manganese-prepared metalloprotease of Bacillus polymyxa. Appl. Microbiol. 26:191–249.

7. Keay, L., P. Moser, and B. Wildi. 1970. Proteases of the genus Bacillus. II. Alkaline proteases. Biochem. Bioeng. 12:213–249.

8. Keay, L., and B. Wildi. 1970. Proteases of the genus Bacillus. I. Neutral proteases. Biochem. Bioeng. 12:179–212.

9. Kingan, S., and J. Ensign. 1968. Isolation and characterization of three autolytic enzymes associated with sporulation of Bacillus thuringiensis var. thuringiensis. J. Bacteriol. 96:629–638.
10. 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.
11. Martin, R., and B. Ames. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. 236:1372–1379.
12. Morihara, K. 1974. Comparative specificity of microbial proteinases. Adv. Enzymol. 41:179–243.
13. Nakata, H., and H. O. Halvorson. 1960. Biochemical changes occurring during growth and sporulation of Bacillus cereus. J. Bacteriol. 80:801–810.
14. Prestidge, L., V. Gage, and J. Spizizen. 1971. Protease activities during the course of sporulation in Bacillus subtilis. J. Bacteriol. 107:815–823.
15. Rogoff, M., and A. Yousten. 1969. Bacillus thuringien-
sis: microbiological considerations. Annu. Rev. Micro-
biol. 23:357–386.
16. Schaeffer, P. 1969. Sporulation and the production of antibiotics, exoenzymes, and exotoxins. Bacteriol. Rev. 33:48–71.
17. Shapiro, A. L., E. Vinuela, and J. Maizel. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem. Biophys. Res. Commun. 28:815–820.
18. Weinberg, E. 1970. Biosynthesis of secondary metabolites: roles of trace metals. Adv. Microb. Physiol. 4:1–44.
19. Yousten, A., and M. Rogoff. 1969. Metabolism of Bacillus thuringiensis in relation to spore and crystal for-
mation. J. Bacteriol. 100:1229–1236.