PRODUCTION, PURIFICATION AND PROPERTIES OF *BACILLUS THURINGIENSIS* NEUTRAL PROTEASE

by

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LIST OF ABBREVIATIONS

Acetyl-tyrosine ethyl ester	ATEE
Benzolyarginine ethyl ester	BAEE
Bromphenol blue	BPB
Bovine serum albumin	BSA
Carboxymethyl	CM
Casein-casitone-glucose medium	CCG
Diethylaminoethyl	DEAE
Diisopropylfluorophosphate	DFP
Ethylenediaminetetraacetic acid	EDTA
Glucose-yeast extract-salts medium	GYS
Morpholinopropane sulfonic acid	MOPS
Nutrient sporulation medium	NSM
Phenylmethylsulfonylfluoride	PMSF
Protease production medium	PPM
Sodium dodecyl sulfate	SDS
Tetramethylethylenediamine	TEMED
Trichloroacetic acid	TCA
Tris(hydroxymethyl)aminomethane	TRIS
Yeast extract medium	YE

INTRODUCTION

Bacillus thuringiensis, a representative of the crystalliferous bacilli, has long been recognized as an effective microbial insecticide against various *Lepidoptera* and *Diptera*. The insecticidic nature of the organism is basically associated with the formation of a protein crystal or parasporal body accompanying sporulation (59). The nature of the toxic crystal, namely, the δ-endotoxin, has been extensively reviewed by many researchers, but little information is available about other aspects of the organism. In order to increase utility of *B. thuringiensis* as a biological insecticide, it is necessary to have as much information about nature of the organism as possible. The special importance of the extracellular protease has been suggested by the possible relationship between protease production and sporulation (29,64) as well as by the normal synchrony of crystal protein and spore formation (59). As there have been no reports relating to the production, purification and properties of the protease of *B. thuringiensis* and in view of the fact that the enzyme was produced spontaneously in high yield, it was thus decided to investigate various facets of this extracellular protease.

The early demonstration of the production of extracellular proteases by various microorganisms (27,40) has led to considerable interest not only in the basic enzyme me-

chanisms but also in the industrial applications of the enzymes. Among these are uses in medicine, the food industry, the detergent industry(spot-removing in dry-cleaning), and in leather, textile and photographic processing(8,9,12,18, 32,61). Many of the proteases studied to date have at least some of the properties required for industrial applications. For example, compared to alkaline proteases, the neutral proteases have not played as important a role in detergent application(8,9). But recent studies on B. subtilis enzymes indicate that the neutral protease is a more effective agent in reducing dental plaque in humans than is the alkaline protease.

In this thesis, I will elucidate two major aspects of the extracellular neutral protease of Bacillus thuringiensis var. kurstaki: the nutritional and environmental factors affecting the production of extracellular protease and some physicochemical as well as enzymatic properties of the purified neutral protease.

REVIEW OF THE LITERATURE

The microbial proteases are excreted by certain microorganisms and can be isolated in their active form from the culture filtrates (27). Based purely on their pH preference, the majority of these enzymes can be divided into three broad groups (27,41): (a) acid proteases, which include the Aspergillus saitoi acid protease, Rhizopus chinesis acid protease, and Paecilomyces varioti acid protease, are characterized by low pH activity and stability profiles, insensitivity to metal chelating agents and phosphorylating compounds such as DFP (27). (b) neutral proteases, which exhibit pH optimum near neutrality, are sensitive to metal chelating agents such as EDTA and O-phenanthroline and have been shown to be metalloenzymes (42,68) which possess rather narrowly defined substrate specificity toward peptide bonds and no esterolytic or amidase activity toward most ester and amide substrates (15,16,50,51,69). (c) alkaline proteases, which are best represented by the subtilisins (the extracellular proteases of B. subtilis) (26), have maximum activity at alkaline pH, cleave a wide range of peptide bonds, possess esterase activity, and are sensitive to reagents such as DFP. This sensitivity indicates they are serine proteases similar to trypsin and chymotrypsin (27,31,41).

The neutral proteases have not been extensively studied until recent years. The isolation and description of

these lesser known extracellular enzymes from culture filtrates have been reported in various microorganisms including Bacillus(41), Streptomyces(41), Pseudomonas(41), Aspergillus(41), Serratia(49), and Staphylococcus(2,3). The neutral proteases are primarily metalloproteases, which share a common bond specificity, have pH optimum around pH 7.0, and are sensitive to metal chelating agents such as EDTA and O-phenanthroline. They are not inhibited by DFP or thiol reagents. The substrate specificities of these enzymes were studied with both simple di- and tripeptides and protein substrates. All the studies indicated a substrate specificity which required a hydrophobic amino acid such as leucine or phenylalanine as the amino acid whose amino group was involved in the bond to be cleaved(41). To date, no amidase activity has been exhibited by these enzymes nor has any esterolytic activity been found toward a host of P-nitrophenyl esters or other ester substrates(41). Calcium has been shown to be important for the maintenance of the enzyme stability(23). The major criterion for grouping the neutral proteases together is the requirement of metal for the catalytic activity of the enzyme. The presence of a single atom of zinc per mole of the isolated neutral protease protein has been shown in B. subtilis by McConn et al.(42,68), in B. thermoproteolyticus by Latt et al.(36), in B. megaterium by Keay et al.(33), in B. cereus by Feder et al.(17), in B.

polymyxa by Fogarty et al. (24), in Aspergillus sojae by Sekine (64) as well as in Serratia by Miyata (49) and Aeromonas proteolytica by Griffin et al. (25). In addition to these demonstrations of zinc-containing enzymes by direct metal analysis, there are also reports on the reactivation of chelator-inhibited neutral proteases by divalent metal ions in Streptomyces naraensis (30) and Aspergillus oryzae (5).

Among all the microbial proteases studied, most work has been done with those of genus Bacillus. McConn et al. (42,68) isolated the B. subtilis neutral protease from a crude preparation of the enzyme obtained from fermentation liquors. They used a combination of DEAE-cellulose treatment, ammonium sulfate fractionation, and CM-cellulose chromatography. The B. subtilis var. amylosachariticus enzyme was purified in a similar manner by Tsuru et al. (70) except that Duolite A-2, an anion exchange resin, was used to remove the pigment and α-amylase, and separation of the neutral and alkaline protease was achieved over DEAE-Sephadex A-50. Later, Keay (34) reported the use of hydroxylapatite for the purification of both enzymes. The only other Bacillus neutral protease which has been studied in any depth is a thermostable enzyme, thermolysin, which was isolated by Endo (14) from the culture medium of the thermophilic bacterium B. thermoproteolyticus (14,36). This organism is a strain of B. stearothermophilus. The homogeneity of the

enzyme was demonstrated by electrophoresis, sedimentation, and Sephadex gel filtration(14). It has been claimed that the neutral protease of B. subtilis var. amylosaccharíticus (69,70,71)is different from the B. subtilis(42,71,72)neutral protease in certain properties, although both enzymes apparently have same specificity as thermolysin(15,16,51,69,70, 71)except for its high thermostbility and amino acid composition(52,57). The purification of B. megaterium extracellular neutral protease from culture filtrates by ethanol and ammonium sulfate fractionation and gel filtration over Sephadex G-200 has also been reported by Millet et al.(46, 47). They also showed the similarity in general properties and specificity of this neutral protease to the B. subtilis neutral protease(46,47). Various strains of B. cereus have been shown to produce proteases in culture filtrates with properties which suggested that they were metalloendopeptidases(1,21,22,37,53,56,60). Salter(60)reported the partial purification of neutral protease from B. cereus NCTC 945 by ammonium sulfate fractionation and DEAE-cellulose chromatography. The enzyme had a pH optimum near neutrality and was completely inhibited by 10^{-4} M EDTA and partially inhibited by NaCN and cysteine. Feder et al.(17)further worked with B. cereus NCTC 945 and reported in 1971 the more complete purification of this B. cereus neutral protease from culture filtrates by acetone precipitation, active charcoal treatment, ammonium sulfate fractionation, and chromatography

over hydroxylapatite and DEAE-cellulose. The enzyme has been shown not only to be a zinc-containing neutral protease but also to possess similar specificity to that of the neutral proteases from B. subtilis, B. megaterium, and thermolysin(17). Furukawa et al.(21,22)separated three proteolytic fractions from B. cereus KP931 fermentation beers. One of these fractions, fraction III, was markedly stabilized by calcium and inhibited by EDTA, properties which are shared with the neutral proteases. Very recently, Fogarty et al. (19)reported the purification of the metalloprotease from B. polymyxa by ammonium sulfate fractionation, acetone precipitation, and gel filtration on Sephadex G-100. The metalloprotease of B. polymyxa also showed the properties and specificity similar to the neutral proteases of other members of the genus Bacillus(24).

In most cases, certain Bacillus species produce the extracellular proteases during the post-exponential growth phase. However, some species do produce the enzyme during exponential growth when grown under appropriate conditions (10,11,48). The function of the protease is not known, but its production is correlated with the onset of a high rate of protein turn over(39,54)and of sporulation in certain bacilli(6,66,74). Mutant analysis and genetic studies suggested that the capacity to form an extracellular protease was closely linked genetically or functionally to the

capacity to sporulate (62,66). Prestidge et al. studied the protease activity during the course of sporulation and found that three proteolytic enzymes isolated from B. subtilis exhibited a rapid increase in specific activity at a time coinciding with the appearance of refractile bodies in the cells (58). Although the specific regulatory mechanisms are not known, the production of the extracellular protease in most Bacillus species can be inhibited by high concentrations of glucose or amino acids, or both (37,63,73). Levisohn et al. (37) reported the inhibition of both the protease production and sporulation by incubating B. cereus T cells with a high level of amino acids. Through studying the wild type and mutants capable of sporulating in the inhibitory mixture of amino acids, Levisohn et al. postulated that extracellular protease production is controlled by the level of a repressor which is directly or indirectly a catabolite or biosynthetic intermediate in many pathways. When the rate of growth is reduced below a certain critical level, the amount of this repressor is lowered, and derepression of protease synthesis occurs. They further postulated that the same catabolic or biosynthetic intermediate may also function in the regulation of synthesis of spore structural components (37). The isolation of mutants capable of sporulating but unable to produce the protease (48) and nonsporulating mutants capable of producing the protease

(66) does imply that the two physiological properties are not necessarily coupled. However, the close linked relationship in time between the initiation of formation of the spore and the protease still suggests a model of general metabolic control of protease production closely related to but distinct from the control of spore formation (37).

MATERIALS AND METHODS

Bacterial culture. Bacillus thuringiensis var. kurstaki (strain HD-1), which was originally isolated by Howard T. Dulmage (13), was obtained from Dr. Terry Couch, Abbott Laboratories, and was used throughout this investigation.

Chemicals and Reagents. Reagent grade chemicals were used throughout this investigation. No chemicals were tested for homogeneity or further purified.

Cupric sulfate, 3,5-dinitrosalicylic acid, glycine, hydrochloric acid, potassium phosphate (dibasic anhydrous & monobasic crystal), phenol reagent 2N solution (Folin-Ciocalteau), sodium carbonate, sodium hydroxide, sodium potassium tartrate, sodium phosphate (dibasic & monobasic), Tris(hydroxymethyl)aminomethane (TRIS), trichloroacetic acid (TCA), and trisodium citrate were obtained from Fisher Scientific Co. (Fairlaw, N. J.)

Acetyl-tyrosine ethyl ester (ATEE), azoalbumin, benzoylarginine ethyl ester (BAEE), bovine serum albumin (BSA), bovine hemoglobin, Coomassie brilliant blue R, ethylenediaminetetraacetic acid (EDTA), dodecyle sodium sulfate (SDS), morpholinopropane sulfonic acid (MOPS), O-phenanthroline, ovalbumin, pepsin, phenylmethylsulfonylfluoride (PMSF), and trypsin were obtained from Sigma Chemicals Co. (St. Louis, Mo.)

Calcium acetate (powder), calcium chloride dihydrate,

manganous chloride, magnesium chloride, and potato starch were obtained from B & A (Baker & Adamsom, Morristown, N. J.).

Acrylamide, ammonium persulfate crystal, bis-acrylamide, 2-mercaptoethanol, and N,N,N',N'-tetramethylethylenediamine (TEMED) were obtained from Eastman Kodak Co. (Rochester, N. Y.).

Acetic acid (glacial), ammonium sulfate, and sulfosalicylic acid were obtained from J. T. Baker Chemical Co. (Phillipsburg, N. J.).

Maltose, nutrient broth (control #569339), nutrient agar, and soluble starch were obtained from Difco Laboratories (Detroit, Mich.).

Glycerol was obtained frome Nutritional Biochemicals Corp. (Cleveland, Ohio).

Acetone was obtained from Ashland Chemical Co. (Columbus, Ohio).

Bromphenol blue (BPB) was obtained from Polysciences inc.

Hydroxylapatite (Bio-Gel HTP) was obtained from BIO-RAD Laboratories (Richmond, Calif.).

DEAE-cellulose was obtained from Whatman, W & R Balston Ltd, Maidstone, Kent, England.

Sephadex G-100 (medium) was obtained from Pharmacia Fine Chemicals, Inc. (Piscataway, N. J.).

Growth conditions. Stock cultures of B. thuringiensis var. kurstaki were prepared by streaking NSM slants, incubating for 20 hours at 30 C and refrigerating the screw cap tubes.

Inocula for growth curve experiments were prepared by the "active culture" technique of Nakata and Halvorson (55). A loopful of the stock culture was placed in a 125 ml. cotton-stoppered flask containing 10 ml. sterile nutrient broth and incubated static at 30 C overnight. One ml. of the overnight broth culture was then transferred aseptically into another 125 ml. cotton-stoppered flask containing 10 ml. sterile nutrient broth. This flask was put on a New Brunswick model G76 gyrotory water bath shaker at 32.5 C and 220 rpm for 3 to 4 hours. Identical transfers were repeated twice more at 3 hour intervals to obtain very young broth inocula for starting the growth curve experiments.

Growth curves were initiated by inoculating 50 ml. of NSM broth contained in a 500 ml. flask with 5 ml. of broth from the last flask in the "active culture" sequence. The flask was shaken at 220 rpm, 32.5 C on a New Brunswick model G76 gyrotory shaker.

Growth was followed by measuring the absorbance in a Klett-Summerson photoelectric colorimeter which was set at zero absorbance (660 nm) with nutrient broth. Cuvettes were 12.5 mm diameter.

Two ml. samples to be used for protease assay were

withdrawn at each hour of growth after inoculation. The bacteria were removed by centrifugation with an IEC-20A centrifuge (Damon/IEC Divison) at 27,000 x g, 4 C for 15 minutes. The culture supernatants were frozen until used for determination of protease activity. Experiments established that no decline in activity took place during several weeks of freezing.

The pH of the growth medium was measured hourly with a Corning pH meter.

Spore formation in relation to the course of growth and production of protease was investigated by drawing 1 ml. samples hourly, sonicating the samples for 1 minute to declump the cells, heating at 80 C for 12 minutes, and diluting and plating in nutrient agar. Samples for spore counts were taken when slightly refractile prespores were first detected by phase contrast microscopy.

Composition of protease production medium (PPM). The NSM broth, which was used in the growth curve experiment, was originally formulated by Fortnagel and Freese (20). This medium was prepared by autoclaving 0.4 g. of nutrient broth in 49.75 ml. of distilled water. When the medium had cooled to below 50 C, 0.25 ml. of autoclaved metal mixture was added. The metal mixture contained 0.14 M $CaCl_2.2H_2O$, 0.01 M $MnCl_2.4H_2O$, 0.2 M $MgCl_2.6H_2O$.

As a result of an examination of the effect of metals

on protease production by B. thuringiensis, the following medium was devised from NSM for production of the highest levels of protease activity. It consisted of nutrient broth (8.0 g/1,000 ml.), 7 x 10^{-3} M $CaCl_2.2H_2O$, 5 x 10^{-4} M $MnCl_2.4H_2O$, and 1 x 10^{-3} M $MgCl_2.6H_2O$. The nutrient broth and the stock metal salts solutions were autoclaved separately and mixed together after cooling.

Preparation of a large volume of crude protease for purification. Two hundred ml. of PPM in each of twelve 2l. flasks was inoculated with 10% (v/v) of B. thuringiensis culture prepared by the "active culture" technique as previously described. The flasks were shaken on the G25 gyrotory shaker at 32 C and 220 rpm for 12 hours. Bacterial cells were removed by centrifugation in a Sorvall RC2-B refrigerated centrifuge using the GSA rotor at 23,300 x g for 20 minutes at 5 C. The resulting culture supernatant containing the crude enzyme was stored at 5 C.

Protease assay. Proteolytic activity was determined by a slight modification of the method of Hanson (28). A 0.5% (w/v) solution of azoalbumin was prepared in a appropriate buffer (0.1 M) at the desired pH.

The reaction mixture contained 1.0 ml. of azoalbumin and 0.7 ml. of distilled water. The reaction was initiated by the addition of 0.3 ml. of the enzyme preparation. After incubating in a water bath at 30 C for 1 hour, the reaction

was stopped by the addition of 2 ml. of 8% TCA solution. The precipitate was removed by centrifugation at 27,000 x g for 15 minutes at 4 C in a IEC-B20 centrifuge. Two ml. of the supernatant was mixed with 2 ml. of 0.5 N NaOH, and the color development was measured spectrophotometrically at 440 nm in a Hitachi Perkin-Elmer Model 139 UV-VIS spectrophotometer with a 1 cm cuvette. A blank was prepared by adding TCA to the substrate prior to the addition of the enzyme solution. When very active enzyme preparations were assayed, they were diluted sufficiently to assure that the resulting absorbance at 440 nm reflected an excess of substrate to enzyme. One unit of protease activity was defined as that amount of enzyme which caused an increase in absorbance at 440 nm of 0.01 per 1 hour under the condition of the test. Specific activity is expressed as units of protease activity per mg of protein.

Determination of amylase activity. Amylase activity was determined by the method of Bernfield (4) using 1% soluble starch in 0.02 M potassium phosphate buffer, pH 6.9, as the substrate solution. The dinitrosalicylic acid reagent was prepared by dissolving at room temperature 1 g of 3,5-dinitrosalicylic acid in 20 ml. of 2 N NaOH and 50 ml. distilled water, adding 30 g of Rochelle salt (sodium potassium tartrate) and making up to 100 ml. with distilled water.

A 1 ml. amount of properly diluted enzyme preparation was incubated for 3 minutes at 25 C with 1 ml. of the substrate solution. The enzymatic reaction was interrupted by the addition of 2 ml. of dinitrosalicylic acid reagent. The tube containing this mixture was heated for 5 minutes in a boiling water bath and then allowed to cool to room temperature. After addition of 20 ml. of distilled water, the absorbance of the solution was determined spectrophotometrically at 540 nm. The blank was prepared in the same manner without enzyme. A calibration curve established with maltose (0.2 to 2 mg. in 2 ml. of distilled water) was used to convert the spectrophotometer readings into mgs. of maltose. One unit of amylase activity is defined as that amount of enzyme which liberates 1 mg. of maltose in 3 minutes at 25 C. Specific activity is expressed as units of amylase activity per mg. of protein.

Determination of esterase activity. Esterase activity was determined by a modification of the method of Prestidge et al. (58). Hydrolysis of benzoylarginine ethyl ester (BAEE) or acetyl—tyrosine ethyl ester (ATEE) was measured at room temperature with a Hitachi Perkin-Elmer Model 139 UV-VIS spectrophotometer. The reaction mixture contained 0.8 ml. of 0.1% BAEE or 0.05% ATEE prepared in 0.1 M pH 7.0 MOPS buffer, 2.1 ml. of the MOPS buffer, and 0.1 ml. of the enzyme preparation. The reference cuvette contained all

components of the assay except enzyme. BAEE hydrolysis was indicated by an increase in absorbance at 254 nm and ATEE hydrolysis by a decrease in absorbance at 237 nm.

Protein determination. Protein was measured by the method of Lowry et al. (38). Bovine serum albumin was used as the standard.

Protease purification. A large volume of culture supernatant containing the crude enzyme, which was prepared as previously described, was concentrated about 20-fold by ultrafiltration with an Amicon stirred cell (Model 52) using a Diaflo ultrafilter UM10 membrane at 5 C with 55 psi nitrogen gas pressure. The concentrate was clarified by centrifugation in a Sorvall RC2-B refrigerated centrifuge using the SS34 rotor at 27,000 x g for 20 minutes.

Solid ammonium sulfate was slowly added with constant stirring to the concentrated crude enzyme solution. At each desired level of saturation, the mixture was allowed to stand 10 minutes to allow precipitation. Fractions of 0-30%, 30-45%, 45-60%, 60-75%, and 75-90% were collected by centrifugation. The precipitates were dissolved in 0.1 M MOPS-0.1% calcium acetate buffer, pH 7.0. The fraction with the highest protease specific activity was then dialyzed against the same buffer at 4 C for 6 hours with one change of the buffer after 3 hours.

Amylase was removed by adding 10% pulverized potato

starch to the most active dialyzed ammonium sulfate fraction in the presence of 12% cold ethanol at 5 C. After 10 minutes stirring, the starch was removed by filtration under vacuum at 5 C. Fresh starch was added and the treatment repeated.

The protease was further purified by adding 2 volumes of precooled acetone to the starch filtrate. After stirring for 5 minutes, the precipitate was collected by centrifugation and washed with acetone/0.1% calcium acetate solution (2:1). The precipitate was redissolved in 0.1 M MOPS-0.1% calcium acetate solution (pH 7.0) and stored at 5 C for application on a hydroxylapatite column.

Hydroxylapatite previously equilibrated with 0.1% calcium acetate, was poured to form a 1 x 4 cm column. The column was cooled to 5 C in the cold room. The enzyme preparation derived from acetone treatment was applied to the column. The column was then washed with about 200 ml. 0.1% calcium acetate at a flow rate of 40 ml./h. Potassium phosphate buffer (pH 7.2) ranging in concentration from 0.01 M to 0.2 M was used to elute the protein. Fractions of 4 ml. were collected at a flow rate of 40 ml./h and were read at 280 nm to determine the protein absorbance. The fractions with protein absorbance were assayed for protease activity.

SDS-polyacrylamide gel electrophoresis and estimation of molecular weight. The apparatus used was the Electro-

phoresis unit obtained from Hoefer Scientific Instrument Inc. The detailed procedures for performing electrophoresis were obtained from Dr. R. C. Bates with a slight modification.

(a) Cleaning of tubes: All the tubes were soaked in dichromate-sulfuric acid for 4 to 6 hours, rinsed very well with distilled water and dried in an oven. They were then washed with 0.1% SDS solution and again dried.

(b) Buffer for electrophoresis: 0.1 M sodium phosphate buffer (pH 7.2) was prepared in large volume as a stock. The working standard phosphate buffer was prepared freshly just before electrophoresis by adding 0.1% SDS powder to the stock buffer.

(c) Preparation of polyacrylamide gels: Reagents used were (i) Solution A: 20 g acrylamide and 0.54 g bis-acrylamide in 100 ml. working standard buffer. (ii) Solution B: 0.15 g ammonium persulfate and 0.1 ml. TEMED in 100 ml. working standard buffer (prepared freshly). The 7.5% gels were prepared by mixing 3 volumes of solution A with 1 volume standard working buffer and then adding an equal volume of solution B. This was mixed well and used to fill the tubes, the bottoms of which were sealed by serum-stoppers. Tubes were filled to 9.5 cm with a Pasteur pipette, and a few droplets of distilled water added to the top to ensure a flat surface. Polymerization was for 20 to

30 minutes. After the gels were hard, the water was shaken off the top of the gels.

(d) Electrophoretic prerun: The bottom tray was filled with an appropriate amount of standard working buffer (about 400 ml.). After placing the tubes in the electrophoresis unit with adaptors, air bubbles were drawn off the bottom of the tubes with a curled Pasteur pipette. The upper tray was filled with enough standard working buffer to cover all the tubes underneath. The electrophoresis unit was connected to a Beckman Duostat regulated D.C. power supply and run at a constant current of 4 mA per tube at room temperature for 1 to 2 hours.

(e) Preparation of samples for electrophoresis: Standard proteins for molecular weight determination were prepared as stock solutions. One hundred μg of denatured bovine hemoglobin, trypsin, pepsin, and fifty μg of denatured ovalbumin and BSA were applied to the gels.

Samples (not more than 250 μl.) were denatured by adding 1/10 volume of 10% SDS in 0.01 M phosphate buffer (pH 7.2) and 1/10 volume of 10% mercaptoethanol. The above mixtures were boiled for 1 minute and cooled. Before applying to each gel, the denatured samples were mixed with 2 drops of glycerol and 2 drops of BPB tracking dye (prepared by dissolving a tiny bit of BPB powder in about 1 ml. of water).

(f) Electrophoresis separation run: Each sample was

applied to the top of the gel with a Pasteur pipette. Electrophoresis was performed at room temperature for about 12 hours at a constant current of 4 mA per gel. Samples were run towards the positive pole.

(g) Staining of gels: Protein bands were visualized by fixing the gels in tubes of 20% sulfosalicylic acid for 8 to 12 hours, staining for 13 to 14 hours with 0.25% Coomassie brilliant blue dye, and destaining with 7% acetic acid (v/v) in the Diffusion destainer, Hoefer Scientific Instrument, Inc.

(h) Scanning of gels for molecular weight estimation: The gels for molecular weight estimation were scanned in a Gilford spectrophotometer at 540 nm and the protein bands were recorded on a Gilford recorder 242. The distance from the top of the gel to the main peak (protein band) was measured. The protein standard curve for molecular weight estimation was plotted on a semi-log graph paper with the data of distance in cm. against molecular weight. The spectrophotometer was zeroed at 540 nm with the clear part of the gels.

Analysis of metal ions. All the glassware used in this experiment was washed with 50% nitric acid and rinsed with glass distilled water. The starch filtrate enzyme preparation was precipitated with solid ammonium sulfate to 65% saturation at 5 C. The precipitate was redissolved in

0.01 M MOPS-0.1% calcium acetate buffer, pH 7.0, and dialyzed against the same buffer for 5 hours at 5 C. The dialyzed enzyme preparation was then applied to a 1 x 12 cm. Sephadex G-100 column previously equilibrated with 0.01 M MOPS-0.1% calcium acetate buffer, pH 7.0. The protein was eluted by washing the column with 0.01 M MOPS-0.1% calcium acetate buffer (pH 7.0) at a flow rate of 40 ml./h. Fractions of 4 ml. were collected and measured for protein absorbance at 280 nm. Those fractions with protein absorbance were assayed for protease activity and the zinc content was determined by atomic absorption spectrometry using the Unican SP90A atomic absorption spectrophotometer, Philips Electronic Instruments. Standard curves were prepared by using zinc reference solution from Fisher Certified Reagent Solution.

The effect of pH upon activity of the protease. 0.5% azoalbumin solutions were prepared in 0.1 M Na.citrate-citric acid buffer for pH 5.5-6.0, in 0.1 M MOPS buffer for pH 6.5-7.0, in 0.1 M TRIS-HCl buffer for pH 7.5-8.5, and in 0.1 M glycine-NaOH buffer for pH 9.0-10.5.

The protease assay was performed and protease activity determined as previously described.

The effect of inhibitors on protease activity. 0.05 M EDTA, 0.001 M O-phenanthroline, and 0.001 M PMSF were the inhibitors used in this experiment. The reaction mixture

contained 0.3 ml. enzyme preparation, 1.0 ml. azoalbumin solution (pH 7.0), 0.1 ml. of 0.1 M EDTA or 0.02 M PMSF or 0.02 M O-phenanthroline, and 0.6 ml. of distilled water. The assays were performed as previously described.

Effect of temperature on enzyme stability. 0.3 ml. aliquots of the enzyme preparation were each incubated at various temperature for 10 minutes prior to the normal protease assay. After these heat treatments, the glass centrifuge tubes with the 0.3 ml. aliquots were immediately cooled in an ice bath. The assays were then performed as previously described. The remaining activities were expressed as percentages of the initial activity.

RESULTS

Optimum pH for protease assay. In view of the lack of knowledge concerning the number and type of protease enzymes present in the culture supernatant, it was first necessary to determine the activity at a variety of pH values. The examination of protease activity with a native crude enzyme preparation was performed by using azoalbumin solutions prepared in different buffers to give a pH range from pH 5.5 to 10.5. This investigation not only established an optimum pH for the azoalbumin assay in measuring protease activity but also gave some information about the number of enzymes present. Figure 1 illustrates the effect of pH on the protease activity under the conditions of the assay. The results indicated that the optimum pH for protease assay is in the range between 6.5-7.0 with the best activity shown at pH 7.0. Therefore, in all subsequent assays the azoalbumin solution was buffered at pH 7.0 with 0.1 M MOPS buffer. The pH profile also indicated that acid or alkaline protease did not constitute a significant part of the total protease activity.

Protease production during growth and the relationship to spore formation. A growth curve of *B. thuringiensis* was performed in NSM broth by using a 10% v/v inoculum from the last flask in an "active culture" series. The "active culture" series was necessary to assure that the inoculum

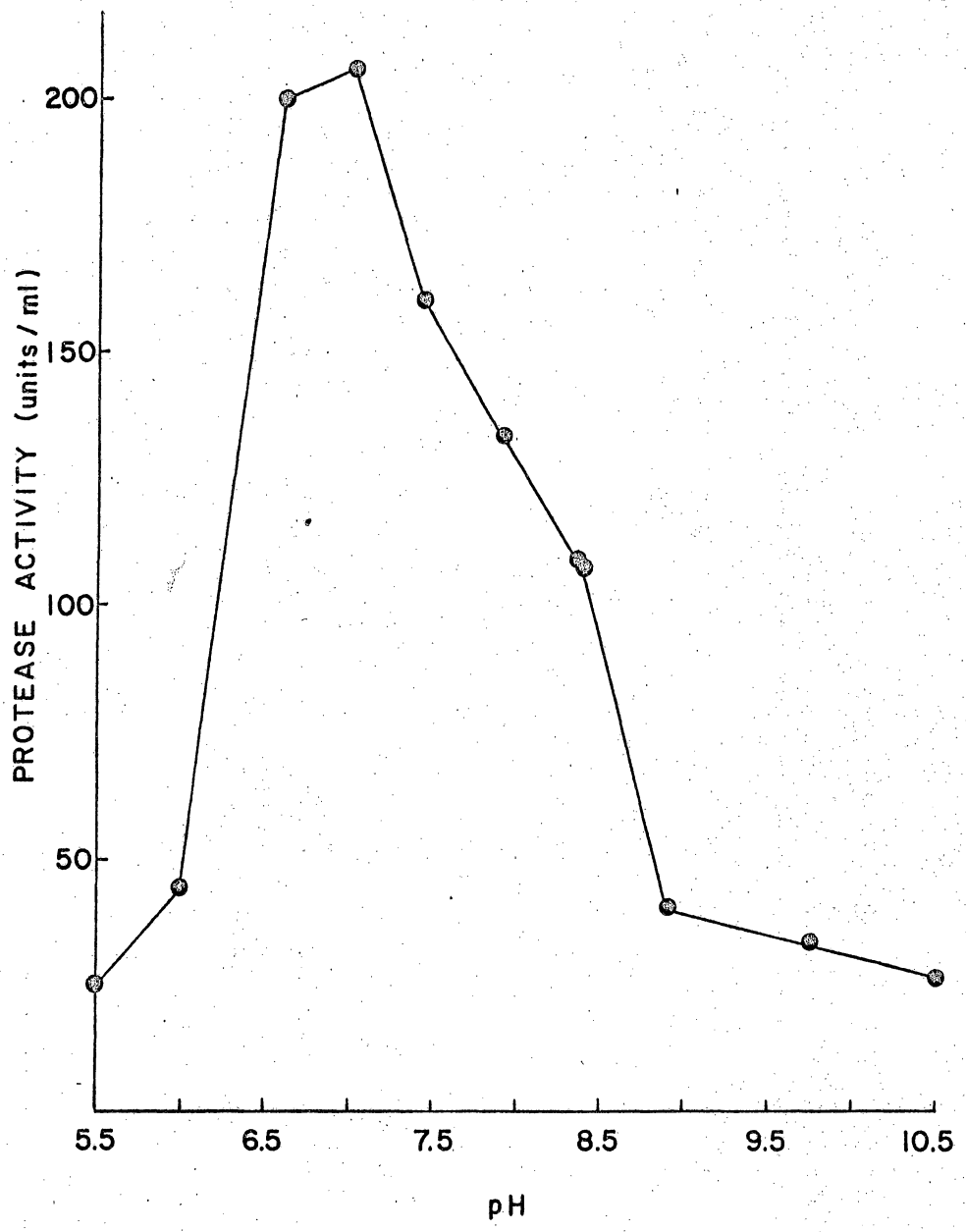

FIGURE 1. Protease activity of B. thuringiensis culture supernatant measured at various pH values.

consisted entirely of physiologically young cells. Growth was followed turbidimetrically until the fifth hour when the cells clumped producing a granular appearance. This made further turbidimetric measurement inaccurate. The pH was measured hourly and 2 ml. samples were taken, centrifuged, and the clear supernatant used for protease assay. When slightly refractile prespores were detected by phase contrast microscopy, sampling was begun to determine the number of heat-stable spores present. Heat-stable spores were those cells which survived a 12 minute treatment at 80 C. The data are presented graphically in Figure 2. Exponential growth of the culture was completed at about the fourth hour. The pH of the NSM broth fell to pH 5.7 at the middle of the exponential phase of growth and then increased again as exponential growth was completed. The pH remained relatively constant in the range of 8.0-8.5 during the stationary phase. Protease production was initiated at the completion of exponential growth and continued for 5 hours. The increase in the number of heat-stable spores coincided with the completion of protease production.

Effect of metal salts on extracellular protease production in nutrient broth. Protease production was first examined by growing B. thuringiensis in various media including GYS (76), CCG (7), YE (7), and NSM. NSM was found to give the best yield of protease. In order to detect the

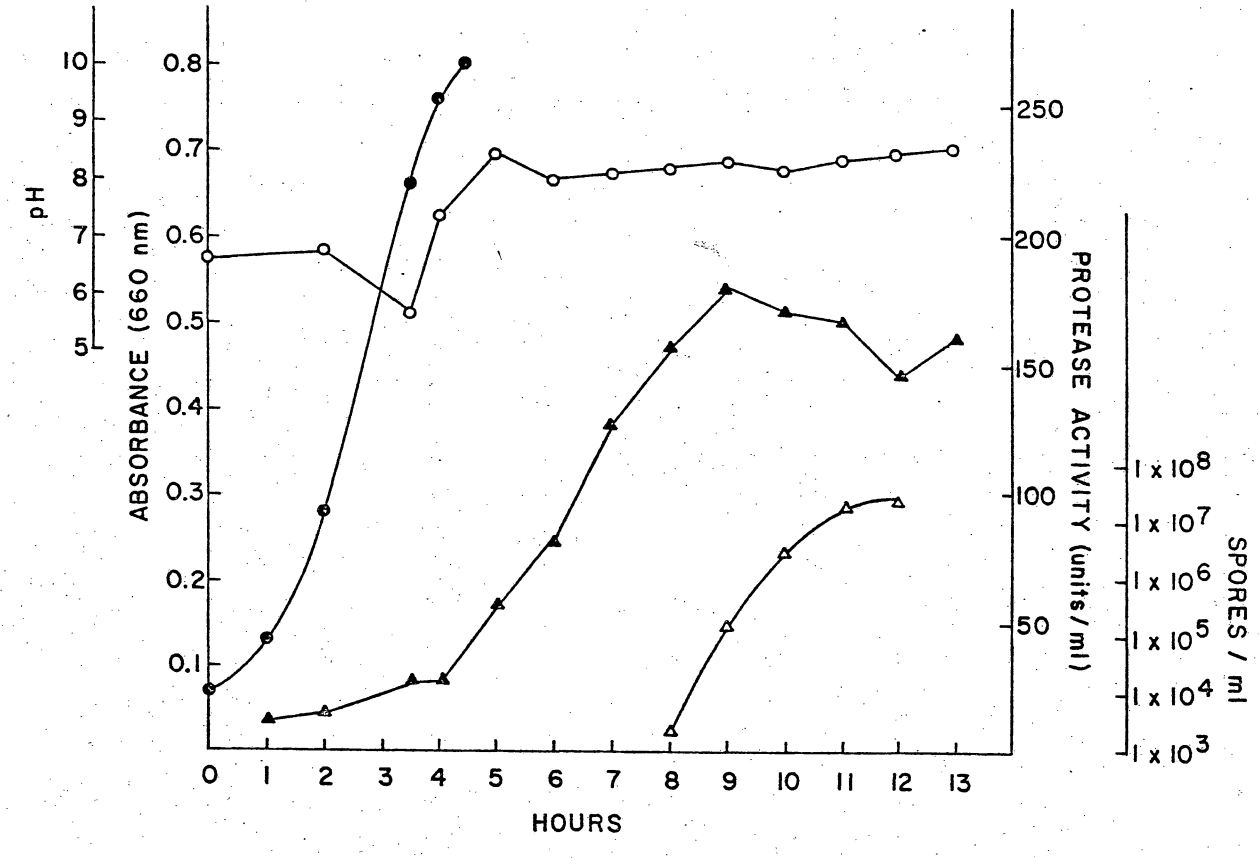

FIGURE 2. Pattern of extracellular protease production, pH changes, and spore formation during the growth of B. thuringiensis. Symbols: ⊙ = absorbance; ○ = pH; ▲ = protease activity; △ = spore count.

effect of the metal salts in the NSM on the production of protease, a series of experiments were done by adding different combinations and concentrations of the three metal salts, namely, calcium chloride, manganous chloride, and magnesium chloride, to the nutrient broth.

The first approach was to add each metal salt individually to nutrient broth in the same concentration as the metal mixture in the NSM, i.e. 7×10^{-4} M $CaCl_2$, 5×10^{-5} M $MnCl_2$, and 1×10^{-3} M $MgCl_2$, and to compare the protease production to that produced in NSM broth. The result is given in Figure 3. In the absence of added metals, i.e. in nutrient broth alone, protease production was low and barely detectable. Of the metal salts added individually, magnesium had the least effect, calcium promoted the formation of a low but readily discernable amount of protease, and manganous ion supplementation allowed formation of almost twice as much protease as did calcium ion supplementation. However, in the presence of manganese alone the protease was unstable and after reaching a peak of activity between 6 and 9 hours, the activity was lost. The combination of all 3 metals at the level found in NSM broth allowed formation of a level of protease activity over twice that found with manganese alone.

It was possible that rather than stimulating synthesis of protease, the metals were merely activating the enzyme in

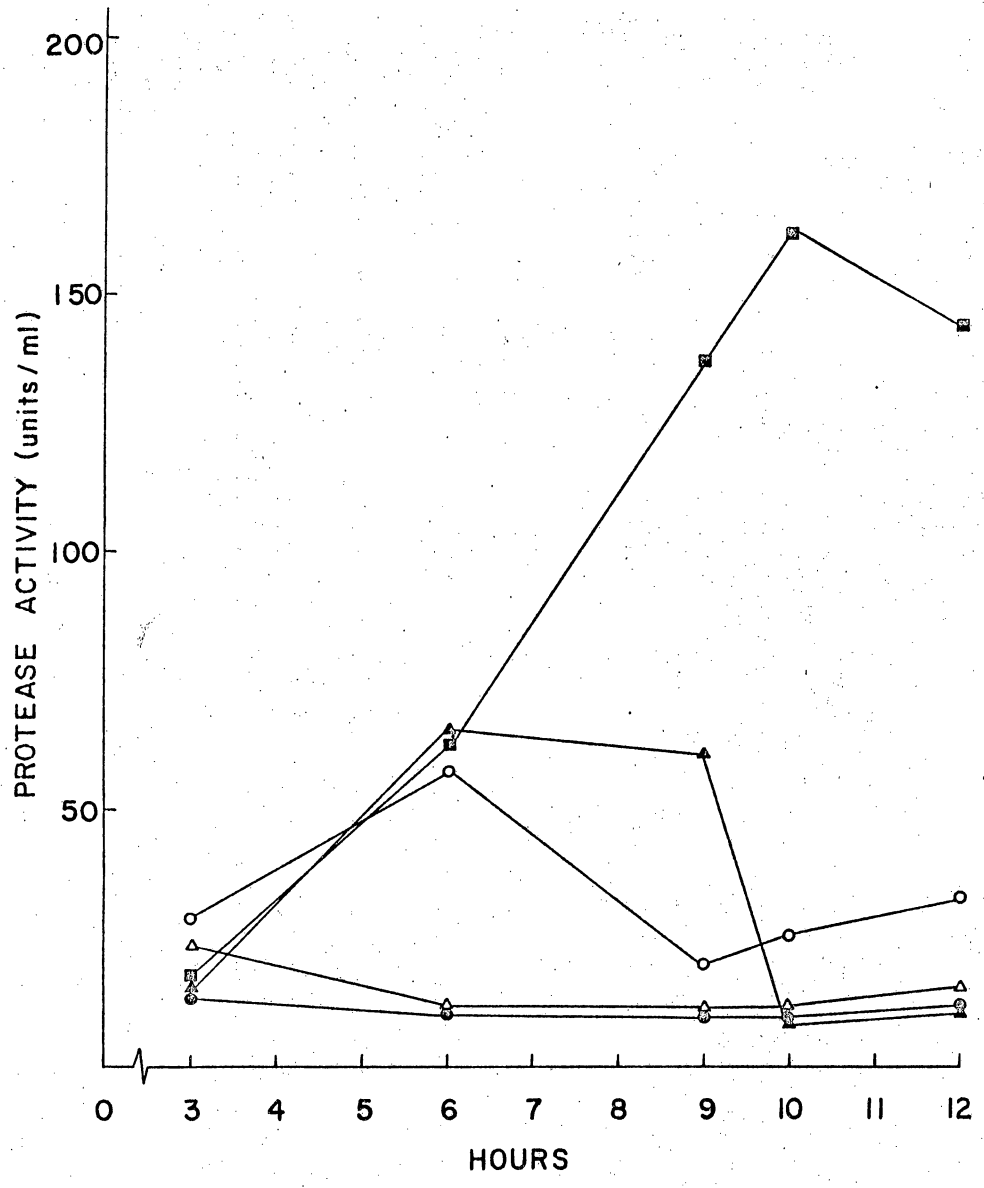

FIGURE 3. Effect of single metal salts on protease production in nutrient broth. Symbols: ⊙ = nutrient broth; ○ = 7×10^{-4} M $CaCl_2$; ▲ = 5×10^{-5} M $MnCl_2$; △ = 1×10^{-3} M $MgCl_2$; ■ = NSM.

the assay system. To test this possibility, B. thuringiensis was grown in nutrient broth, nutrient broth + 5 x 10^{-5} M manganous chloride, and in NSM and 9 hour culture supernatants were assayed with and without metal supplements. The data in Table 1 clearly show that adding metals to the assay system did not stimulate enzyme activity and therefore the effect of metals is definetely to promote enzyme synthesis.

The second approach was to add different combinations of two metal salts to nutrient broth and to compare the protease production to the normal NSM broth. Each salt was in the same concentration found in NSM broth. The results are shown in Figure 4. The combination of Mn^{2+} and Ca^{2+} in nutrient broth allowed better protease production than any of the other paired combinations and this pair even allowed better protease production than found in NSM. Although Mg^{2+} and Mn^{2+} supplementation allowed protease production almost to the level found in NSM, the activity was lost. This points to a role for Ca^{2+} in maintaining enzyme stability. This role of Ca^{2+} was confirmed by the stability of the enzyme produced in Ca^{2+} and Mg^{2+} supplemented broth.

From the above results, the presence of both manganese and calcium in the nutrient broth was found to be essential for both the production and stability of protease. Using this information, the effect of different concentrations of Mn^{2+} and Ca^{2+} in the presence of 1 x 10^{-3} M $MgCl_2$ on protease

Table 1. Effect of added metals on the activity of *B. thuringiensis* protease*

Protease source	Metal salts added to assay mixture	Protease activity (units/ml)	Protease activity obtained from protease assay without metal salts addition (units/ml)
Nutrient broth	7×10^{-4} M $CaCl_2$ 5×10^{-5} M $MnCl_2$ 1×10^{-3} M $MgCl_2$	11.6	10
Nutrient broth + 5×10^{-5} M $MnCl_2$	7×10^{-4} M $CaCl_2$ 1×10^{-3} M $MgCl_2$	8.6	60
NSM	-	139	139

* Culture supernatants used as the source of enzyme in this experiment were 9 h old at the time of harvest.

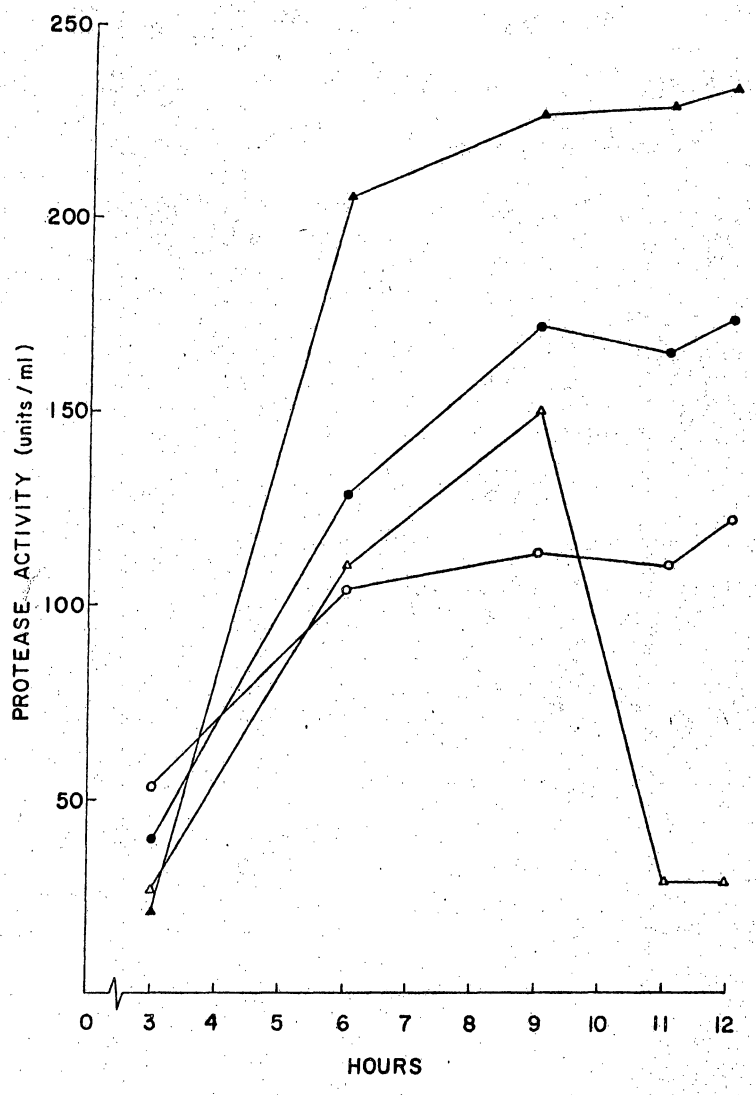

FIGURE 4. Effect of different combinations of two metal salts in nutrient broth on protease production. Symbols: ● = NSM; ○ = 7×10^{-4} M $CaCl_2$ and 1×10^{-3}M $MgCl_2$; ▲ = 7×10^{-4}M $CaCl_2$ and 5×10^{-5}M $MnCl_2$; △ = 5×10^{-5}M $MnCl_2$ and 1×10^{-3}M $MgCl_2$.

production was examined. The result, which is presented in Figure 5, indicates that 7 x 10^{-3} M $CaCl_2$ and 5 x 10^{-5} M $MnCl_2$ as well as 7 x 10^{-3} M $CaCl_2$ and 5 x 10^{-4} M $MnCl_2$ included in in the nutrient broth with 1 x 10^{-3} M $MgCl_2$ were optimum for protease production. The importance of Ca^{2+} for enzyme stability was again clearly demonstrated.

Although Mg^{2+} was included in the media when optimum Ca^{2+} and Mn^{2+} levels were determined (Figure 5), it was possible that this metal could be eliminated from the medium with no less of protease production. This effect was suggested earlier (Figure 4). In the experiment presented in Figure 6, the Mn^{2+} and Ca^{2+} levels were held at 5 x 10^{-5} M and 7 x 10^{-3} M respectively while the Mg^{2+} level varied from none to 1 x 10^{-3} M. The final 12 hour activity indicated that 1 x 10^{-3} M Mg^{2+} allowed more protease production than other levels but the difference from no Mg^{2+} to 1 x 10^{-3} M Mg^{2+} was only about a 15% increase in activity.

Zinc has been shown to be a cofactor required for enzymatic activity of some bacterial neutral proteases (17,24, 33,42,68). However, supplementation of the growth medium with various concentrations of Zn^{2+} did not enhance protease activity (Table 2).

At this point, an experiment was run to see if different combinations of higher concentrations of $CaCl_2$ and $MnCl_2$ in the presence of 1 x 10^{-3} M $MgCl_2$ could give a better yield

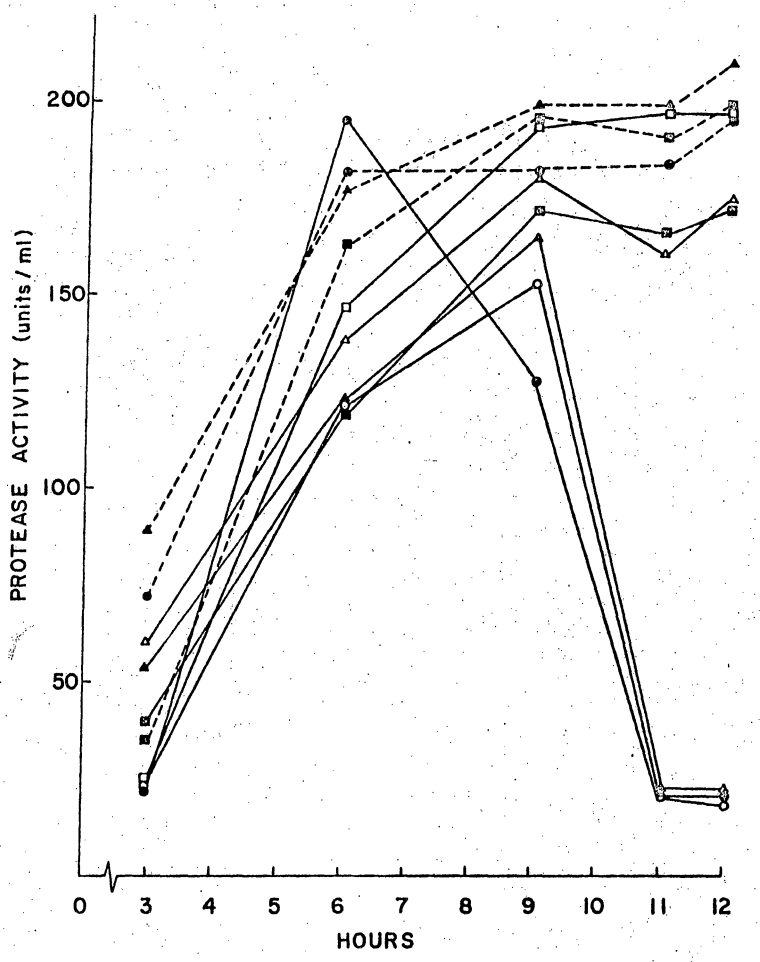

FIGURE 5. Effect of different concentrations and combinations of Mn^{2+} and Ca^{2+} with 1×10^{-3}M Mg^{2+} on protease production.

(●——●) = 7×10^{-5}M $CaCl_2$ + 5×10^{-6}M $MnCl_2$
(○——○) = " " + 5×10^{-5}M $MnCl_2$
(▲——▲) = " " + 5×10^{-4}M $MnCl_2$
(△——△) = 7×10^{-4}M $CaCl_2$ + 5×10^{-6}M $MnCl_2$
(■——■) = NSM
(□——□) = 7×10^{-4}M $CaCl_2$ + 5×10^{-4}M $MnCl_2$
(●---●) = 7×10^{-3}M $CaCl_2$ + 5×10^{-6}M $MnCl_2$
(▲---▲) = " " + 5×10^{-5}M $MnCl_2$
(■---■) = " " + 5×10^{-4}M $MnCl_2$

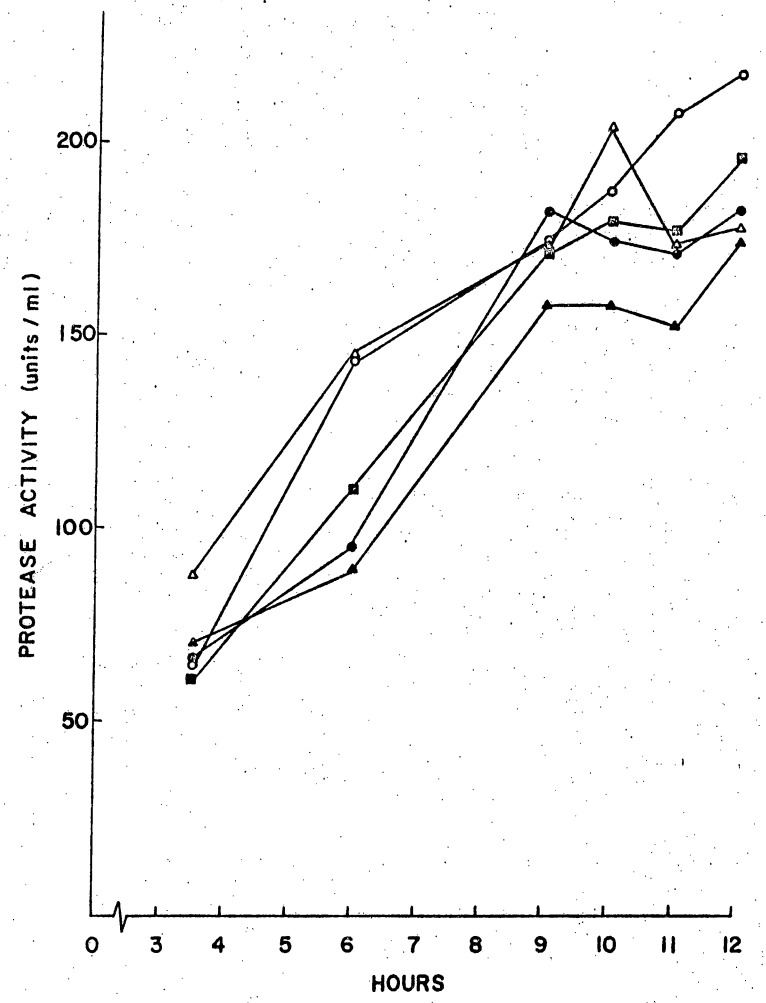

FIGURE 6. Effect of different concentrations of $MgCl_2$ in nutrient broth containing 7×10^{-3} M $CaCl_2$ and 5×10^{-5} M $MnCl_2$ on protease production. Symbols: ● = no $MgCl_2$; ○ = 1×10^{-3} M $MgCl_2$; ▲ = 1×10^{-4} M $MgCl_2$; △ = 1×10^{-5} M $MgCl_2$; ■ = 1×10^{-6} M $MgCl_2$.

Table 2. Effect of different concentrations of Zn^{2+} in the nutrient broth containing 7×10^{-3} M $CaCl_2$, 5×10^{-5} M $MnCl_2$,and 1×10^{-3} M $MgCl_2$.

Hours of growth	Protease activity (units/ml)			
	No $ZnSO_4$	1×10^{-6} M $ZnSO_4$	1×10^{-5} M $ZnSO_4$	1×10^{-4} M $ZnSO_4$
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

of protease. The data from this further experiment is exhibited in Figure 7. The best protease production during the growth of B. thuringiensis was in a nutrient broth containing 7×10^{-3} M $CaCl_2$, 5×10^{-4} M $MnCl_2$, and 1×10^{-3} M $MgCl_2$. This particular medium which was very favorable for the production of protease was named "Protease Production Medium" (PPM) and was used throughout the remainder of the investigation.

Purification of the extracellular protease. The purification procedures described in Materials and Methods for the B. thuringiensis protease are similar to those used by Keay et al. (35) for the B. subtilis NRRL B3411 neutral protease. Table 3 lists steps used in enzyme purification and the results obtained at each step. Following growth of the organism, the cell-free culture supernatant was concentrated using an Amicon ultrafiltration cell with a UM10 membrane having an exclusion limit of approximately molecular weight 18,000. After concentration the total amount of protein present decreased about 68% but there was a small increase (1.83 x) in specific activity. After adding 2% calcium acetate powder, further purification was achieved by use of ammonium sulfate fractionation. Of the fractions taken at various percentages of saturation (see Materials and Methods, page 17), the 45-60% fraction contained most of the activity and represented a 67-fold increase in specific activity

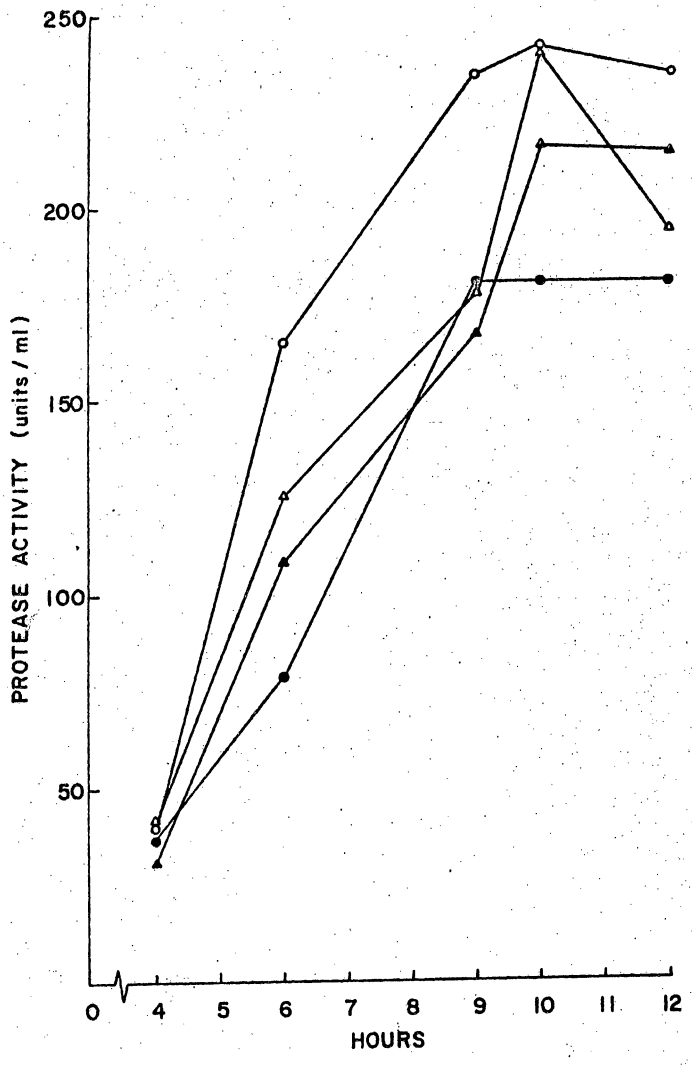

FIGURE 7. Protease production during the growth of B. thuringiensis in different concentrations and combinations of $CaCl_2$ and $MnCl_2$ with 1×10^{-3}M $MgCl_2$ in nutrient broth. Symbols: ● = 7×10^{-3}M $CaCl_2$ + 5×10^{-5}M $MnCl_2$; ○ = 7×10^{-3}M $CaCl_2$ + 5×10^{-4}M $MnCl_2$; ▲ = 7×10^{-2}M $CaCl_2$ + 5×10^{-5}M $MnCl_2$; △ = 7×10^{-2}M $CaCl_2$ + 5×10^{-4}M $MnCl_2$.

Table 3. Purification of *Bacillus thuringiensis* exoprotease

Steps	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity (u/ml)	Total activity (u)	Specific activity (u/mg)	Purification (fold)	Enzyme yield (%)	Amylase activity (u/ml)	Amylase Specific activity (u/mg)
Crude enzyme solution	2,400	1.85	4,440	190	456,000	102.7	1	100	1.2	0.65
Ultrafiltration cell concentrate	122	11.7	1,427.4	2,200	268,400	188	1.83	58.85	15.75	1.34
Ammonium sulfate fractions 45-60%	18	1.85	33.3	12,666	227,988	6,846.5	66.67	50	20	10.8
60-75%	18	1.05	18.9	700	12,600	666	6.4	2.7	-	-
Dialyzed ammonium sulfate fraction (45-60%)	16	1.7	27.2	12,666	202,656	7,451	72.55	44.4	21.25	12.5
Starch treated enzyme filtrate	14	1.0	14	10,000	140,000	10,000	97.37	30.7	0.375	0.375
Acetone treated fraction	10.5	0.6	6.3	4,800	50,400	8,000	77.9	11	-	-
Hydroxylapatite chromatography	9	0.06	0.54	430	3,870	7,166.7	70	0.84	-	-

compared to the enzyme obtained from the crude enzyme preparation. The main contaminating enzyme at this point was believed to be amylase which had itself been purified 10 x by these procedures (Table 3). Following dialysis to remove ammonium sulfate, the 45-60% ammonium sulfate fraction was treated with pulverized potato starch in the presence of 12% ethanol. The starch with adsorbed amylase was removed by filtration under vacuum. This treatment removed about 97% of the amylase and what appeared to be a significant part of brownish pigment which remained after the ammonium sulfate treatment. At this point, the enzyme specific activity had increased about 97 fold. Further purification using cold acetone resulted in a decrease in activity. Chromatography on hydroxylapatite also resulted in a decrease in activity.

To prepare a quantity of partially purified protease for use in enzyme characterization, the purification scheme used in Table 3 was slightly modified. The acetone precipitation and hydroxylapatite steps were eliminated since these were found to be non-productive. The purification sequence shown in Table 4 was used to prepare enzyme used for molecular weight determination, to determine the effect of pH on activity, to determine the thermal stability of the enzyme, and to determine the relationship of zinc to enzyme activity. This purification resulted in an enzyme preparation in which the specific activity had been increased about

Table 4. Purification of *Bacillus thuringiensis* exoprotease

Steps	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity (u/ml)	Total activity (u)	Specific activity (u/mg)	Purification (fold)	Enzyme yield (%)	Amylase activity (u/ml)	Amylase Specific activity (u/mg)
crude enzyme solution	2,400	1.453	3487.2	212	508,800	145.8	1	100	0.6	0.413
Ultrafiltration cell concentrate	128	11.25	1440	2,283	292,224	202.9	1.39	57.44	5.875	0.527
Ammonium sulfate fractions 0-45%	8	0.5	4	540	4,320	1080	7.4	0.84	-	-
45-60%	20	1.75	35	14,833	296,660	8476	58.1	58.31	12.625	7.21
60-65%	8	0.894	7.15	3,200	25,600	3579.4	24.5	5.03	1.5	1.67
Dialyzed ammonium sulfate fraction (45-60%)	17	1.6	27.2	16,333	277,661	10208.1	70.01	54.57	12.625	7.265
3 x starch treated enzyme filtrate	13	0.872	11.34	11,733	152,529	13455.3	92.29	29.98	0.325	0.372

92 times that in the original culture supernatant.

SDS-polyacrylamide gel electrophoretic analysis of protease purification. To evaluate the purity of the enzyme preparation, crude culture supernatant, concentrated culture supernatant, the 45-60% ammonium sulfate fraction, and the filtrate from starch treatment were subjected to polyacrylamide gel electrophoresis in separate gels. The result is pictured in Figure 8. In gel number 1, 290 µg of protein in the crude culture supernatant gave a single faint band. The concentrated crude enzyme (1125 µg of protein applied to the gel) showed a single dark band (gel 2). Electrophoresis of 262 µg of protein from the 45-60% ammonium sulfate fraction (gel 3) showed a main band located at the same migration distance as the bands in gels 1 and 2. In addition two slower moving bands are present. Gel 4 received 87 µg of the starch treated enzyme preparation. A single band appeared which migrated at a rate equal to the main bands in gels 1, 2 and 3. The appearance of a single main band throughout purification, the increasing specific activity of the protease, and the decreased amount of protein applied to gel 4 point to the main band being the protease. The appearance of 2 other bands in the ammonium sulfate fraction and the disappearance of these bands along with amylase following starch treatment indicate that one of the slowly moving bands in gel 3 may have been amylase. The single strong

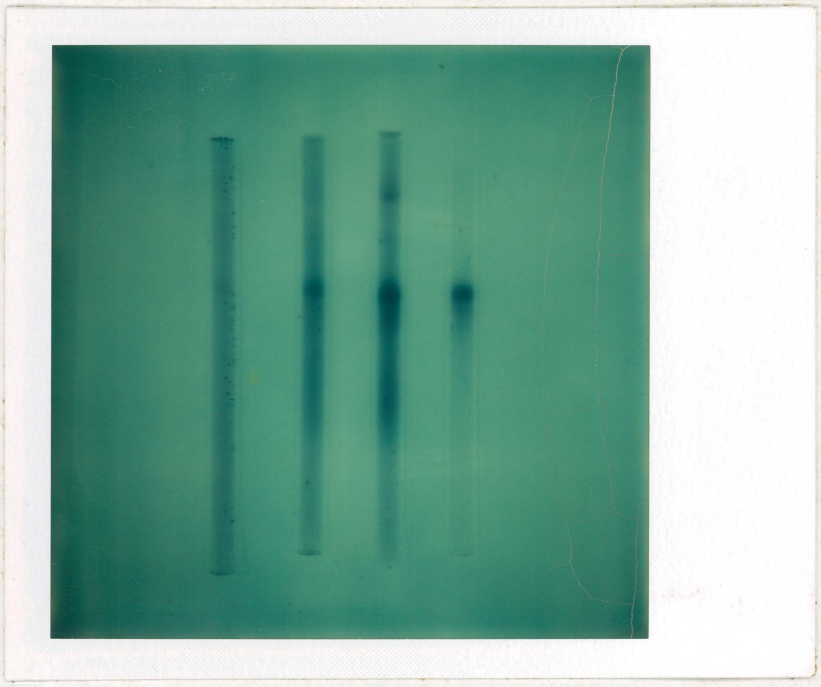

Figure 8. SDS-polyacrylamide gel electrophoresis of enzyme preparations during purification

band in gel 4 indicates that no other protein is present in any quantity approaching that of the protease.

Molecular weight of the B. thuringiensis var. kurstaki neutral protease. The molecular weight of the neutral protease was estimated to be about 37,000. This was done by electrophoresis in SDS-polyacrylamide gels. The reference proteins used for the protein standard curve were bovine hemoglobin (15,500 MW), trypsin (23,300 MW), pepsin (35,000 MW), ovalbumin (43,000 MW), and BSA (68,000 MW). Following electrophoresis for 12 hours at 4 mA per tube the gels were scanned. The distance from the top of each gel to the point of the peak was measured on the chart paper and the standard curve was obtained by semi-log plot of molecular weight against distance of migration in cm. (Figure 9). A line of best fit has been drawn through the points.

The gels with each standard protein and protease are pictured in Figure 10. From left to right are hemoglobin (1), trypsin (2), pepsin (3), protease (4), ovalbumin (5), and BSA (6).

Effect of pH on protease activity. Figure 11 illustrates the effect of pH on enzyme activity of the partially purified protease. The extracellular protease of B. thuringiensis showed a pH optimum near neutrality.

Effect of temperature on stability. Figure 12 shows the thermal inactivation curve of the partially purified

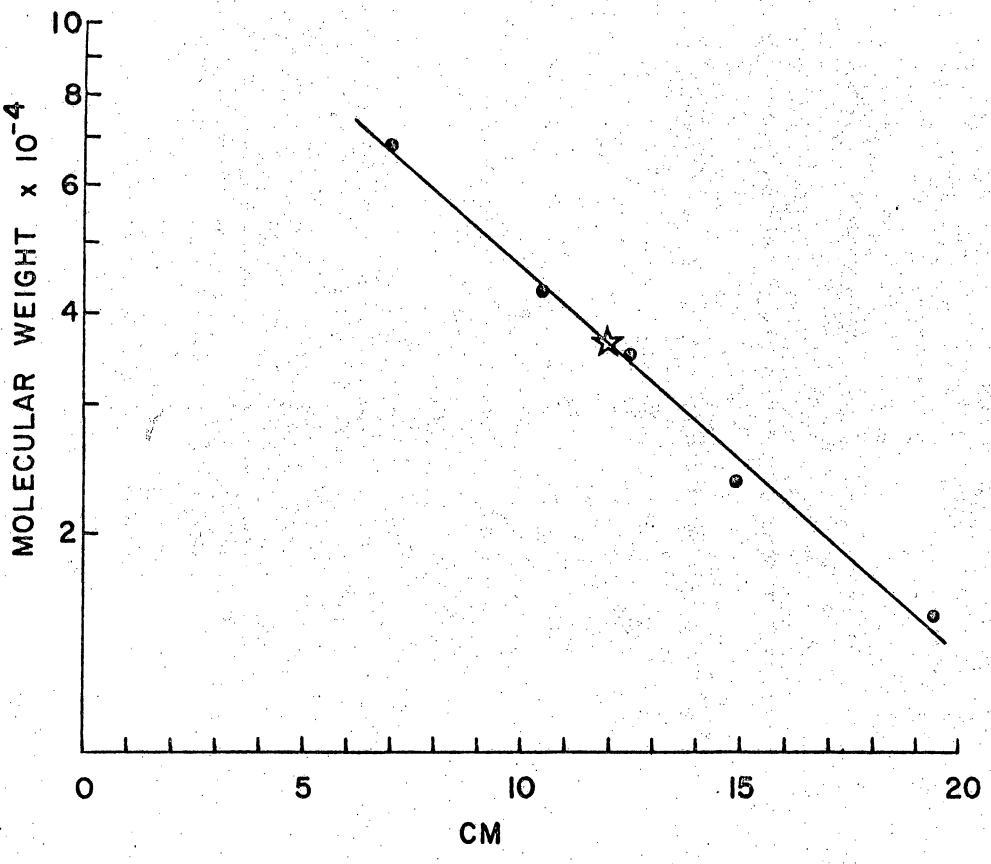

FIGURE 9. Standard curve for molecular weight estimation. ☆ = data point for B. thuringiensis protease.

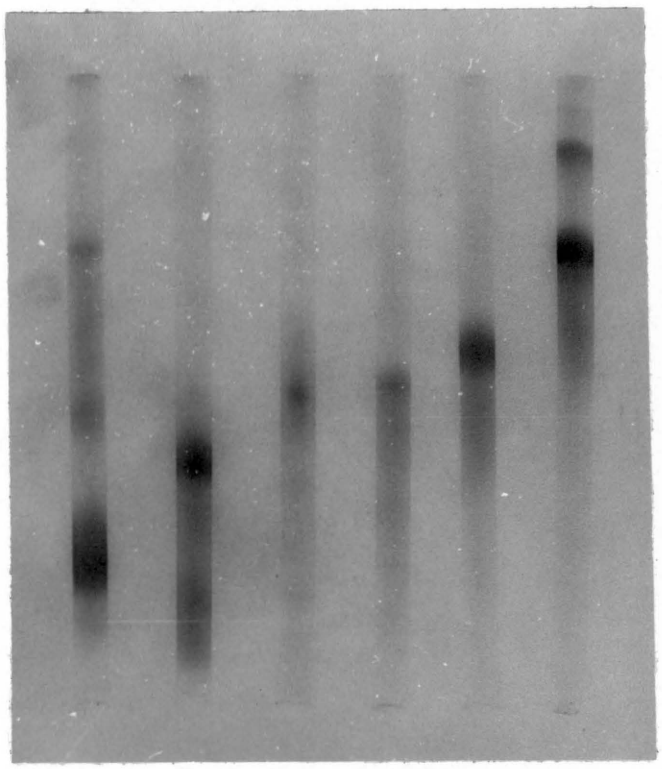

Figure 10. Molecular weight estimation on SDS-polyacrylamide gel electrophoresis.

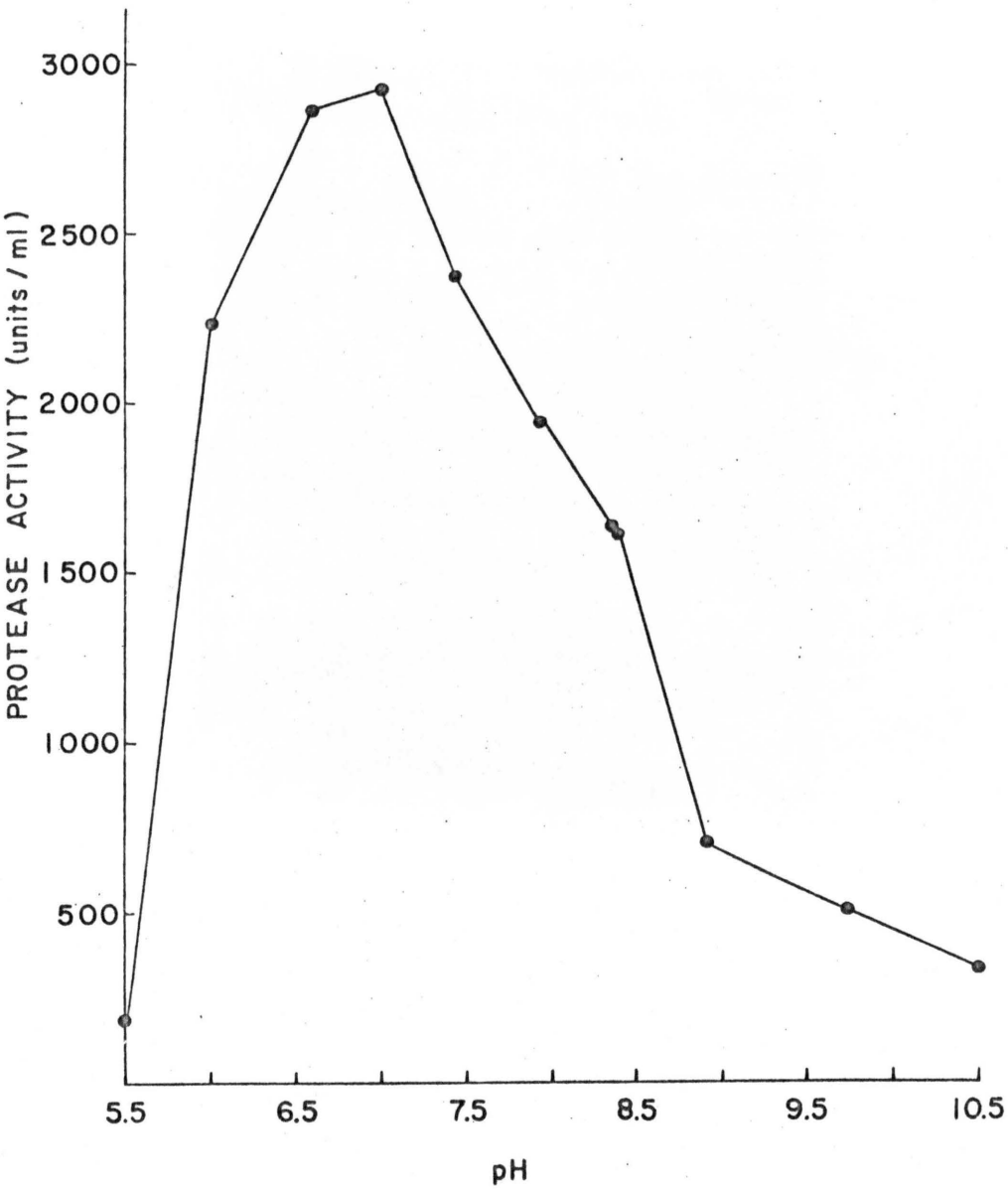

FIGURE 11. Effect of pH on the activity of B. thuringiensis exoprotease.

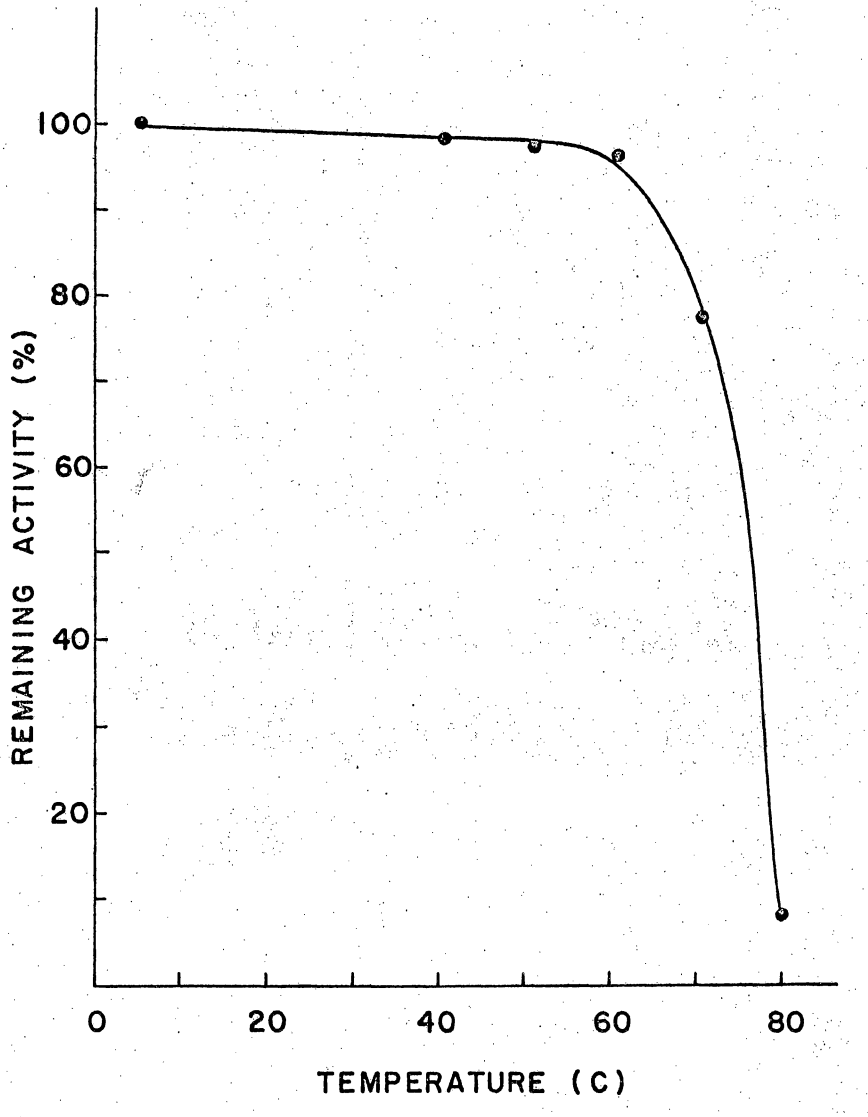

FIGURE 12. Thermal stability of B. thuringiensis neutral protease.

neutral protease. The enzyme was quite thermostable in the presence of 0.1% calcium acetate. The first indication of thermal inactivation was observed when the enzyme was incubated at 70 C for 10 minutes. Ninty percent of the activity was lost following treatment at 80 C for 10 minutes.

Effect of inhibitors on protease activity. The crude enzyme was markedly inactivated by the metal chelating agents, 0.05 M EDTA and 0.001 M O-phenanthroline, as shown in Table 5. Phenylmethylsulfonylfluoride (PMSF), an inhibitor of serine alkaline proteases, showed no inhibitory effect.

Esterase assay. No esterase activity against either BAEE or ATEE substrate was detected with either crude or partially purified enzyme preparation.

Relationship of zinc to protease activity. Studies of neutral proteases from other microorganisms have indicated a relationship between zinc and enzyme activity (17,24,33, 42,68). A possible relationship between zinc and enzyme activity for the B. thuringiensis neutral protease was sought by examining the zinc content of Sephadex G-100 gel filtration fractions which contained protease. The source of enzyme applied to the column was material which had been starch treated to remove amylase (see Table 4) and reprecipitated with 65% ammonium sulfate. The eluting buffer (0.01 M MOPS, pH 7.0, containing 0.1% calcium acetate)

Table 5. Effect of various inhibitors on protease activity

Inhibitor	Final concentration	remaining activity
None	-	100%
EDTA	5×10^{-2} M	11%
O-phenanthroline	1×10^{-3} M	6%
PMSF	1×10^{-3} M	100%

contained no zinc when tested by atomic absorption spectroscopy. All of the protease activity was eluted from the column in 3 four ml. fractions. The zinc to protein ratio in the three fractions was almost constant indicating that the zinc in the effluent was protein associated. However, the zinc to protease activity ratio was not constant indicating that some other protein(s) containing zinc were still present in the partially purified enzyme (Table 6).

Table 6. Relationship of zinc to protease activity.

Sephadex G-100 gel filtration fractions	Protease activity (units/ml)	Protein (mg/ml)	Protease specific activity (units/mg)	Zn^{2+} content (mg/l)	Zn^{2+} / protein	Zn^{2+} / protease activity
1	233.3	0.0615	3793	0.2809	0.0046	1.2×10^{-6}
2	1266.6	0.128	9896	0.5185	0.0041	4.1×10^{-6}
3	175.0	0.0675	2593	0.3293	0.0048	1.9×10^{-6}

DISCUSSION

Protease production in relation to the growth and spore formation of B. thuringiensis. The temporal relationship between spore formation and protease production was one of the earliest indications in the literature that there might be a biochemical relationship between the two events. Although the temporal relationship was very clearly indicated in this study, this is no longer regarded as good evidence of a biochemical connection. This is particularly true in light of the reports by Michel and Millet (45) and Millet and Archer (46) that mutants of B. subtilis and B. megaterium lacking the extracellular neutral protease sporulate normally. In the absence of protease negative mutants of B. thuringiensis, it is not possible to evaluate the possibility of a biochemical connection between protease synthesis and sporulation in this organism. It is quite possible that both are merely catabolite repressible events which occur when that repression is relaxed.

The pH profile found during growth of B. thuringiensis var. kurstaki was similar to that reported earlier for B. thuringiensis var. galleriae (76). Acidic metabolites produced during growth apparently lowered the pH. With the derepression of tricarboxylic acid cycle enzymes, these acids were oxidized and the pH rose.

The role of trace metals in the biosynthesis of extra-

cellular protease. Of the metals included in nutrient broth for protease production by B. thuringiensis, it was found that the yield of protease was most sensitive to the levels of manganese and calcium. Manganous ions were particularly essential for the maximum yield of protease. Calcium ions, on the other hand, had a stabilizing effect on protease activity. Magnesium ions alone were not essential to protease production, but the presence of magnesium ions along with manganese and calcium was shown to give the best enzyme yield.

Stockton et al. (67) in their work with B. subtilis reported the essential role of manganous ions for protease production. Weinberg (75) also indicated that manganese is the "key" metal for species of Bacillus in the synthesis of secondary metabolites. Examples of secondary metabolites whose synthesis depends upon manganese are bacitracin in B. licheniformis, bacillin, D-glutamyl polypeptide, and subtilin in B. subtilis, and phage and spores in B. megaterium (75). Although Weinberg excluded extracellular protease from the group of secondary metabolites, present work does demonstrate the "key" metal effect of manganese upon its synthesis. The precise site of the manganese effect in the scheme of protein synthesis is unknown.

Gorini in 1950 (23) was one of the first to show the importance of calcium in activation and stabilization of

protease. Later, the stabilizing effect of calcium ions on the neutral proteases of B. subtilis (42) and B. megaterium (33) was shown. This effect has now been demonstrated with the B. thuringiensis neutral protease. Without the addition of calcium to the growth medium, protease was produced but was unstable, probably due to self-digestion. The requirement for calcium is aggrevated by the removal of calcium from the medium by the endospore as it acquires heat resistance.

Feder et al. (17) in their study of B. cereus neutral protease indicated that some stabilization of protease was achieved with $MgCl_2$. Although the exact role of magnesium ion in protease production is not clear, the data reported here does demonstrate the necessity of supplying magnesium in the growth medium for optimum protease production.

Purification of the extracellular protease. The pH activity profile of the crude culture supernatant showed an optimum activity at pH 7.0 and very little activity at pH 9.0. This was evidence against the presence of an alkaline protease as is produced by B. subtilis in addition to its neutral protease. Also, the activity of the crude enzyme was inhibited almost completely by chelating agents and not at all by PMSF. This supported the presence of a metal containing neutral protease rather than an alkaline protease with serine at the active site. Thus, at the outset of

purification the evidence pointed to the presence of a single neutral protease.

Use of the Amicon ultrafiltration cell was successful in concentrating the culture filtrate and removing some low molecular weight proteins and other small molecules. Ammonium sulfate fractionation was very successful in achieving a large increase in specific activity. Almost all of the protease was confined to the 45-60% fraction. SDS-polyacrylamide gel electrophoresis at this stage showed two contaminating proteins, one of which was almost certainly amylase. Assays indicated that amylase had been purified along with the protease up to this stage. Repeated treatments with pulverized potato starch and precipitation with cold ethanol were successful in eliminating 95% of the amylase. About a 92 x purification had been achieved at this stage and electrophoresis showed a single band of protein. This does not prove that other proteins were not present, but it does indicate that if present they were present in very low levels. Attempts to further purify the enzyme by cold acetone precipitation and hydroxylapatite chromatography were unsuccessful.

Physical, chemical and enzymatic properties of the B. thuringiensis neutral protease. Estimation of the molecular weight of the purified protease by SDS-polyacrylamide gel electrophoresis gave a value of about 37,000. This falls

within the 35,000-40,000 molecular weight range of neutral proteases produced by most microorganisms reported by Matsubara et al (41).

The purified enzyme exhibited optimum activity in the range of pH 6.5-7.0. This is similar to the sharp peak of pH 7.3 observed for B. megaterium (33) and pH 7.0-7.2 for B. cereus (17). This observation supports the neutral protease nature of the B. thuringiensis enzyme.

The purified protease was quite thermostable in the presence of calcium. Treatment at 70 C for 10 minutes only decreased activity by 23%. As it true for other neutral proteases, the extracellular protease of B. thuringiensis did not show esterase activity against the two substrates tested. This is in contrast to alkaline proteases which often possess esterase in addition to protease activity.

The considerable inhibition of protease activity in the crude enzyme preparation by the metal-chelating agents EDTA and O-phenanthroline and the ineffectiveness of PMSF (an inhibitor of serine alkaline protease) gave not only a further indication of the neutral protease nature of the enzyme but also was indirect evidence of the metallo-protease nature of the neutral protease.

Although the zinc found in Sephadex G-100 column fractions was clearly associated with protein, the zinc to units of protease activity ratio was not constant. Thus it cannot

be conclusively stated that zinc is the metal associated with enzyme activity. Further purification of the enzyme would be necessary to eliminate the apparent presence of one or more contaminating zinc-containing proteins.

It can be concluded that B. thuringiensis is similar to B. cereus, B. megaterium, B. thermoproteolyticus, and B. polymyxa in that it only produces a neutral extracellular protease. Also, the neutral protease of B. thuringiensis is similar to most neutral proteases produced by other microorganisms in that it has a pH optimum near neutrality, it is sensitive to metal-chelating agents, it is insensitive to trypsin inhibitor, and it has no esterase activity.

SUMMARY

1. Upon completion of exponential growth in a metal salts supplemented nutrient broth, B. thuringiensis var. kurstaki (HD-1) synthesized and excreted a single neutral protease. This synthesis was completed prior to the appearance of heat resistant spores.

2. A protease production medium (PPM) was developed which optimized extracellular protease production by B. thuringiensis. PPM contained nutrient broth supplemented with 5×10^{-4} M $MnCl_2$, 7×10^{-3} M $CaCl_2$, and 1×10^{-3} M $MgCl_2$.

3. A simple procedure was developed which allowed a high degree (97 x) of purification of the enzyme.

4. A molecular weight of 37,000 was determined for the partially purified protease by electrophoresis on SDS-polyacrylamide gels.

5. The pH optimum of the extracellular protease activity was determined to be about pH 6.5-7.0.

6. The partially purified neutral protease lacked esterase activity against BAEE and ATEE.

7. The neutral protease was shown to be inhibited by the metal-chelating agents, EDTA and O-phenanthroline, but not by PMSF, a trypsin inhibitor.

8. The zinc content in relation to the protease activity was investigated.

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PRODUCTION, PURIFICATION AND PROPERTIES OF *BACILLUS THURINGIENSIS* NEUTRAL PROTEASE

by

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(ABSTRACT)

Although the insect pathogenicity of *Bacillus thuringiensis* is reasonably well understood, relatively little is known about other facets of this microorganism's physiology. At the time of sporulation, the organism produces in addition to the spore and toxic paraspore (crystal) an extracellular proteolytic enzyme. This study concerns the conditions for production, the purification and the properties of this enzyme.

It was found in studies relating to production of protease that *B. thuringiensis* var. *kurstaki* (HD-1) produced a considerable quantity of the enzyme in a protease production medium (PPM). This medium contained 7×10^{-3} M $CaCl_2$, 5×10^{-4} M $MnCl_2$ and 1×10^{-3} M $MgCl_2$ in nutrient broth. Manganese was required for enzyme synthesis and calcium was required for enzyme stability.

Starting with a large volume of crude enzyme preparation obtained from the culture supernatant of *B. thuringiensis* grown in PPM, the enzyme was purified 97 x. The purification steps included Amicon ultrafiltration cell concentration, ammonium sulfate fractionation, and potato starch adsorption. Electrophoresis on SDS-polyacrylamide gels showed

a single protein band at the last purification step.

The enzyme had a pH optimum around pH 6.5-7.0 and was sensitive to metal chelating agents such as EDTA and O-phenanthroline. The molecular weight of the neutral protease has been estimated to be about 37,000 by electrophoresis in SDS-polyacrylamide gels. In the presence of 0.1% calcium acetate, the enzyme is quite stable at 60 C after 10 minutes incubation. It lacks esterase activity when tested against acetyl-tyrosine ethyl ester and benzoylarginine ethyl ester. All the above properties indicate the similarity of the Bacillus thuringiensis neutral protease to those produced by other members of the genus Bacillus as well as to the other microbial neutral proteases.