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Protease Activities During the Course of Sporulation in *Bacillus subtilis*

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Three proteolytic enzymes have been isolated from sporulating cultures of *Bacillus subtilis*. These activities were, respectively, a protease inhibited by ethylenediaminetetraacetic acid (EDTA) but not phenylmethylsulfonyl fluoride (PMSF), a protease active on both protein and ester substrates, and an ester-active enzyme with low activity on proteins. The latter two enzymes were inhibited by PMSF but not by EDTA. The specific activity of each was determined both intra- and extracellularly during growth and sporulation in a single-defined medium. All three enzymes were shown to exhibit a rapid increase in specific activity at a time coinciding with the appearance of refractile bodies in cells.

Regulatory systems involved in bacterial sporulation have been the focus of considerable interest in recent years (11, 17). The elucidation of such controls in bacteria, if discovered, could provide a useful model for the broader problems of mammalian cell differentiation. From the mass of accumulated biochemical and genetic data, a concept has emerged which views sporulation as an ordered sequence of biochemical events in which, at some point in time, commitment becomes irreversible (6). A number of biochemical events, such as antibiotic and protease synthesis as well as changes in activity of tricarboxylic acid cycle enzymes, have been implicated in early phases of sporulation (8, 19).

A more detailed examination of these early events has become possible with the demonstration that "protease activity" phenotype is actually a complex consisting of at least two separate activities (3, 14). A third enzyme found extracellularly was reported by Millet (15). Studies by Balassa (1) suggest that a number of proteolytic activities remain to be characterized. In the present investigation, three proteolytic activities were demonstrated in sporulating cultures. The time sequence of formation of these proteases was followed in relation to other sporulation events. Future studies of mutants blocked in any one of these three activities should prove a useful first step in establishing the role of proteases in sporulation.

MATERIALS AND METHODS

Bacterial strains utilized were the parent organism *Bacillus subtilis* 168 trp2$^-$ and a high protease producing (hpr) mutant hpr-97trp2$^-$ derived from it by ultraviolet (UV) irradiation. In transduction experiments the point mutation of hpr-97 was located between *his*A1 and *arg*C4 on the chromosome (9).

Inocula for growth were prepared from cultures grown 48 hr on AK agar no. 2 plates (BBL) supplemented with 0.005 M $MgSO_4$. Spores and cells obtained from plate growth were suspended in water and washed twice by centrifugation. Concentrated suspensions in water were heat-shocked twice (15 min at 70 C) and stored at 4 C prior to use.

Growth medium, a modification of the medium of Donnellan et al. (5), contained $MgCl_2$, 10^{-3} M; KH_2PO_4, 3.8×10^{-4} M; NaH_2PO_4, 1.2×10^{-4} M; Na_2SO_4, 7.75×10^{-6} M; $CaCl_2$, 10^{-4} M; $FeCl_3 \cdot 6H_2O$, 3.48×10^{-6} M; NH_4Cl, 10^{-2} M; $MnCl_2 \cdot 4H_2O$, 10^{-4} M; NH_4NO_3, 1.2×10^{-3} M; casein hydrolysate (Nutritional Biochemicals Corp.), 0.2%; sodium lactate (Fisher Scientific Co.), 0.72%; glucose, 0.05%; L-tryptophan, 20 μg/ml, *p*H 7.6.

Growth and sporulation were followed in a 14-liter capacity fermentor (Fermentation Design Inc., model 1125). Aeration was maintained at 8 liters^3/min, agitation at 300 rev/min, and temperature at 37 C. A suspension of approximately 10 to 15 ml of heat-shocked spores was used to inoculate a 10-liter culture at an initial optical density (OD at 660 nm) of 0.030 to 0.060. OD, *p*H, and refractile body formation in the culture were followed in samples removed after various intervals of time. Per cent of cells containing refractile bodies was the average value obtained from estimates by two independent microscopic observations. Total viable cells were measured by colony formation after portions of culture (suitably diluted in minimal salts; reference 18) were spread on Tryptose Blood Agar Base (TBAB, Difco) plates. Heat-resistant spores were those giving rise to colonies on TBAB after the initial (10^{-2}) dilution tube was subjected to a heat shock of 15 min at 70 C.

Extracellular proteolytic activities and initial characterization studies were carried out on material concen-

trated by either of the methods outlined below.

(i) Thirty liters of 24- to 36-hr-old cultures were freed of cells by centrifugation in a Sharples centrifuge at 40,000 to 50,000 rev/min. The cell-free supernatant fluid was then concentrated approximately 10 times in a Sears humidifier (model 7292) at room temperature. Further concentration at this step was avoided since it caused precipitation of salts from the medium. The concentrate was adjusted to *p*H 8.0 with KOH, and solid $(NH_4)_2SO_4$ was slowly added to 65% saturation. After overnight storage at 4 C, the flocculent precipitate was collected by centrifugation at 9,000 rev/min for 60 min at 4 C (Sorvall model RC2 centrifuge, type GSA rotor). Considerable material was lost at this step in decanting supernatant liquid from the loosely packed precipitates. Final recoveries were generally about 30 to 50% of the activity on azocasein found in the starting supernatant fluid. Collected precipitates from a number of separate experiments were dissolved in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, *p*H 8.0, and the solution was dialyzed against Tris before lyophilization and storage at −20 C.

(ii) A 5 to 10 times enrichment of proteolytic activity was obtained through storage of cell-free supernatant fluid at −20 C and subsequent collection of the liquid first thawing on exposure to room temperatures. Such "freeze-thaw" concentrates were dialyzed and stored at −20 C. Assays on a number of different substrates indicated that the initial proportion of activities was retained in the concentrates. Recovery on the order of 10% of the total activity present was obtained in each thawing cycle. Thus, total recovery was sacrificed for rapid and relatively gentle concentration by this method.

Intracellular proteolytic activities were determined after breakage of cell pellets obtained from samples of culture centrifuged at 3,000 rev/min for 5 min at room temperature (Sorvall Angle centrifuge, type SP rotor). Tubes containing resuspended cell pellets were maintained in an ice bath during breakage by sonic treatment (three exposures for 1 min each at setting 8 on a Bronson model S 125). The broken cell suspensions were freed of cell debris by centrifugation at 15,000 rev/min (Sorvall centrifuge, type SS-34 rotor) for 15 min and then dialyzed prior to assay.

Assays. Protein was determined by modification of the method of Lowry et al. (12) by using bovine albumin fraction V as standard.

Dipicolinic acid was measured by the procedure of Janssen et al. (10) in samples removed from cell suspensions after sonic treatment and prior to centrifugation. Protein in these samples was determined on material precipitated by trichloroacetic acid prior to the assay. This procedure was carried out to avoid false values due to contamination of cell pellets by traces of medium.

Azocasein hydrolysis was measured at 30 C in a reaction mixture containing 0.3 ml of enzyme sample in buffer, 0.1 ml of 1 M Tris-hydrochloride, *p*H 8.0, either 0.1 ml of water or 0.1 ml of M ethylenediaminetetraacetic acid (EDTA), and 0.5 ml of 2% azocasein (Pierce). After suitable incubation times (generally between 15 and 90 min, depending on expected activity), the reaction was stopped by the addition of 2 ml of cold 7% perchloric acid, and tubes were centrifuged 5 min at 5,000 rev/min (Sorvall Angle centrifuge, type SP rotor). Decanted supernatant fluid was made alkaline by the addition of 0.3 ml of 10 N NaOH and the OD at 430 nm was determined. OD readings were linear both with respect to enzyme concentration and time of incubation up to values of 0.600. Azocasein units were defined as the change in absorbancy at 430 nm per 60 min per ml under the conditions described above.

Hydrolysis of benzoylarginine ethyl ester (BAEE, Mann Fine Chemicals, Inc.), ATEE (acetyl-tyrosine ethyl ester, Calbiochem), and *p*-nitrophenylacetate (Sigma Chemical Co.) was carried out at 30 C in a Gilford recording spectrophotometer with a reference cuvette containing all components of the assay except enzyme. The wide range of activity found in crude cell extracts compared to partially purified enzymes necessitated very different full-scale values of the recorder (relative OD) and chart speeds (relative time). Thus, activity results (OD/time) given in the text are plotted in arbitrary units not comparable from one figure to another.

BAEE reaction was indicated by an increase in OD at 254 nm. The reaction mixture contained 1.9 ml of extract in buffer; 0.3 ml of 2 M glycine-NaOH buffer, *p*H 9.05; 0.8 ml of 0.86 mg of BAEE per ml in 0.01 M Tris-hydrochloride, *p*H 8.0. ATEE hydrolysis was measured as an OD at 237-nm decrease in a reaction mixture composed of 1.7 ml of enzyme extract in buffer; 0.3 ml of 2 M glycine-NaOH buffer, *p*H 9.05; and 1.0 ml of 0.5 mg of ATEE per ml in 0.01 M Tris-hydrochloride, *p*H 8.0. *p*-Nitrophenylacetate breakdown was measured by decreased absorption at 405 nm in a reaction mixture containing 0.1 ml of enzyme extract in buffer; 0.6 ml of 0.5 mg/ml *p*-nitrophenylacetate in 0.043 M sodium phosphate, *p*H 7.1, plus 14.3% methanol; and 2.3 ml of sodium phosphate plus methanol buffer.

Column chromatography. Sephadex G-75 fractionation was carried out in a system equilibrated with 0.1 M Tris-hydrochloride, *p*H 8.0, containing 0.2 M NaCl to negate ion-exchange effects reported for Sephadex resins at low ionic strength (7).

Hydroxyapatite (HA, Bio-Rad) was suspended in 0.005 M sodium phosphate, *p*H 7.1, and fines were decanted two or three times. Columns were poured over an initial layer of acid-washed sand about 0.5 cm thick and rinsed with 2 to 3 column volumes of 0.05 M $CaCl_2$ to maximize capacity (16). After equilibration with 0.005 M sodium phosphate, *p*H 7.1, containing 0.2 M NaCl, samples which had been dialyzed in the same buffer were applied. A gradient of sodium phosphate was used to elute adsorbed proteins.

RESULTS

Separation and initial characterization of proteases. Among the mutants isolated in this laboratory is a class designated *hpr* which shows elevated protease activity as measured by zones of clearing on azoalbumin and casein agar plates. One of these mutants, hpr-97, was used in these experiments.

Glucose is known to repress formation of a number of vegetative cell enzymes and has been

shown to affect protease activities during sporulation (4). To avoid increased synthesis of proteases attributable solely to glucose exhaustion, it was necessary to devise a medium where the energy source for final cell growth was derived from some other carbon source. Sodium lactate (final concentration, 0.72%) proved suitable for this purpose. A considerably lower concentration of glucose (0.05%) was added to facilitate outgrowth after germination of the spore inoculum. The glucose was exhausted several hours prior to final cell divisions on lactate as indicated by growth curves. Spore inocula were used to reduce the initial proteolytic activity associated with vegetative cells and to insure greater reproducibility from one experiment to another. Germination, growth, and sporulation were all carried out in a single medium to avoid artifacts caused by centrifugation or other transfer techniques.

Cell-free supernatant liquid obtained from cultures of hpr-97 grown for 24 to 36 hr was used as starting material for protease purification. Maximal activities were reproducibly obtained under these conditions. Concentrates were obtained either by ammonium sulfate precipitation or freeze-thaw techniques. Both of these procedures yielded material from which three major components having proteolytic activity could be isolated by column chromatography.

Fractionation primarily on the basis of molecular weight was first carried out on Sephadex G-75. Fractions were tested for activity on a number of substrates and three main peaks of activity were found. Figure 1 shows the activity profile obtained on the substrates BAEE and azocasein. The first two peaks to be eluted were reproducibly obtained and had considerable activity on Benzoyltyrosine ethyl ester (BTEE, Calbiochem) and ATEE as well as BAEE. The third peak to be eluted was a distinctly different enzyme which hydrolyzed ATEE and azocasein but was not appreciably active on either BAEE or BTEE.

Identical separation on G-75 was obtained with starting material from cultures of the parent strain, *B. subtilis* 168 trp2$^-$. However, relative quantities of the three enzymes differed from those found with mutant hpr-97. Proteolytic activities from strain 168 were not subjected to the further separation methods used for strain hpr-97, and therefore it cannot be stated that the proteases in these two strains behave identically.

The two peaks of BAEE activity (obtained by G-75 fractionation of material from hpr-97) appeared to have different molecular weights. However, both peaks were assumed to contain the same enzyme since they had identical activity ratios on a number of substrates, had very similar heat-inactivation curves, and were negatively

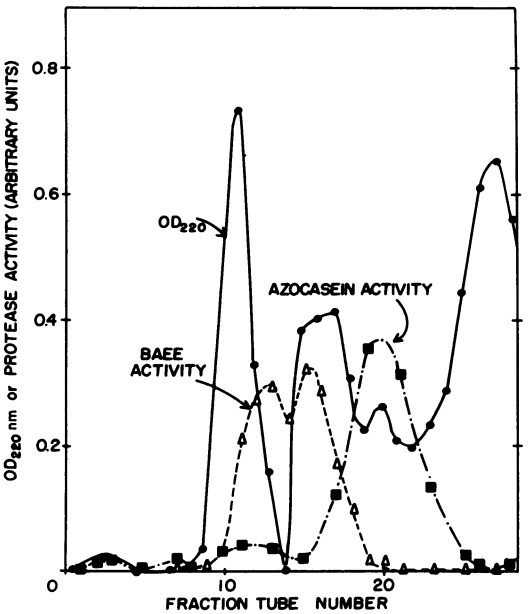

FIG. 1. *Fractionation of protease activities on Sephadex G-75. Lyophilized material obtained from ammonium sulfate precipitation of supernatant fluid from hpr-97 cultures was dissolved in 0.1 M Tris, pH 8.0, plus 0.2 M NaCl. A sample of approximately 14 mg (dry weight) was applied to a Sephadex G-75 column (1.5 cm diameter and 42 cm height), and fractions of 2.5-ml volume were eluted. Samples of fractions were assayed for activity on the substrates BAEE and azocasein. The results are plotted as activity (in arbitrary units) vs. fraction tube number.*

charged as evidenced by binding to diethylaminoethyl (DEAE) cellulose at *p*H 7.1. At *p*H 8.0, used in elution from G-75, either aggregation or binding to positively charged residues present in crude starting material may have caused the appearance of these two peaks of apparently different molecular weights.

On the other hand, the third peak of azocasein activity found on G-75, originally assumed to contain a single protease, was later demonstrated to contain two separate proteolytic activities. Combined fractions of this peak which had been stored at 4 C proved to have altered ATEE-azocasein activity ratios when reassayed several weeks later. It was also noted that ATEE activity was unaffected by EDTA although azocasein activity was considerably reduced.

A number of column chromatographic procedures were tested and some of the earlier inconsistencies were finally resolved when separation of all three proteases was achieved on HA. As Fig. 2 indicates, two activities (A and B) passed through HA unadsorbed. However, B must have been partially held back under the conditions

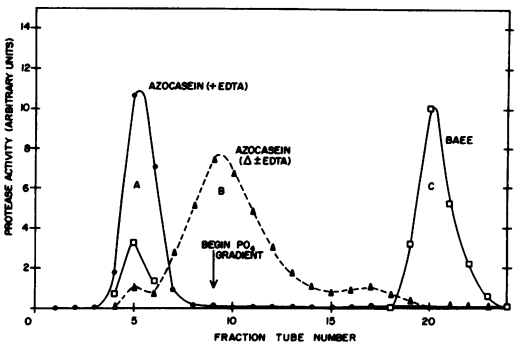

FIG. 2. *Separation of proteases on hydroxyapatite. A freeze-thaw concentrate of supernatant fluid from a culture of hpr-97 at t_8 was dialyzed against 0.005 M sodium phosphate buffer, pH 7.1, plus 0.2 M NaCl. A sample of 7.5 ml (containing 4.2 mg of protein) was applied to an hydroxyapatite column (2.0 cm diameter and 4.5 cm height), and fractions of 4.4-ml volume were collected during elution with 0.005 M phosphate plus 0.2 M NaCl. At tube no. 9 a linear gradient of phosphate in 0.2 M NaCl was started. Enzyme C was eluted at an estimated phosphate concentration of 0.1 M. Activities found in the fractions are plotted (in arbitrary units) against fraction tube number.*

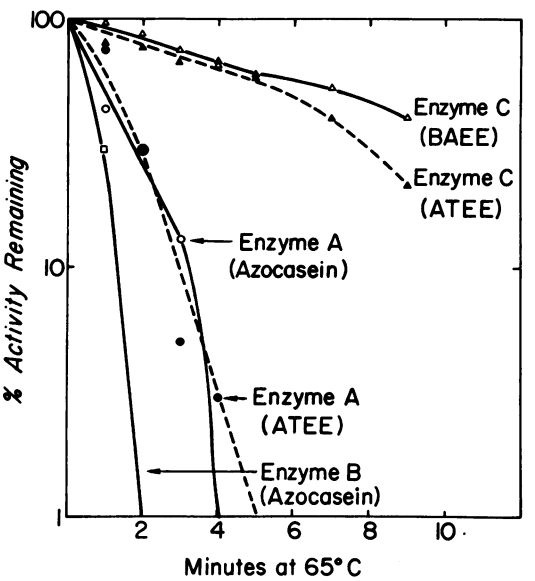

FIG. 3. *Heat inactivation of protease activities. Enzyme preparations were dialyzed against 0.005 M sodium phosphate buffer, pH 7.1, and samples were transferred into a series of test tubes which were immersed at the same time in a water bath at 65 C. At various time intervals, tubes were removed and cooled at once in an ice bath. The per cent activity found in heated samples compared to an unheated control is plotted (on a logarithmic scale) versus time of heating (on a linear scale).*

used since it was separated from A. The third enzyme, C, was adsorbed on HA but could be eluted at approximately 0.1 M sodium phosphate in a gradient subsequently applied.

Although the major portion of BAEE activity (enzyme C) was consistently adsorbed on HA, a fraction of this activity, on occasion, passed through the column and was found associated with peak A. Successive rechromatography decreased the amount of BAEE activity which was not adsorbed. These results were interpreted similarly to those obtained with Sephadex G-75 fractionation, i.e., enzyme C (BAEE activity) is either found as an aggregate or is bound to other compounds in crude preparations which prevents adsorption on HA. Although the evidence is not conclusive, the activity was judged to be due to a single molecular species.

A brief survey of the properties of these proteases was next carried out. For this purpose enzyme B was used directly after separation on HA, whereas both enzymes A and C were adsorbed and eluted from ion-exchange resins at *p*H 7.1 as an additional purification step. For enzyme A, Bio-gel CM-30, a cation exchanger was utilized, whereas enzyme C was purified on DEAE cellulose, anion exchanger. Enzyme A is thus a positively charged molecule; enzyme C is negatively charged at *p*H 7.1.

After the relative heat stabilities of the three enzyme preparations at 65 C were determined, results of a heat-inactivation curve (Fig. 3) indicated that enzyme B was extremely heat labile, whereas enzyme A was somewhat more resistant. Enzyme C was relatively resistant since approximately 39% of the initial activity was still present after 9 min at 65 C.

Results of qualitative tests of activity on various substrates and the effect of some inhibitors and metal ions are presented in Table 1. Enzyme C appeared to be active on a number of synthetic esters not hydrolyzed by either A or B, whereas the latter two enzymes were considerably more active on protein substrates. Both enzymes A and C were inhibited by phenylmethylsulfonyl fluoride (PMSF) and therefore presumably contain a serine residue in their active sites. Inhibition of enzyme B by EDTA indicated a metal ion requirement, although stimulation of activity of enzyme C by EDTA may suggest its greater sensitivity to traces of inhibitory metal ions. Dialyzed preparations of B proved extremely unstable, even when stored frozen, but some activity could be restored by several hours of exposure to Ca^{2+} or Zn^{2+} prior to activity assays. Optimum *p*H for both enzymes A and C was found to lie at the highest point of the range tested (6.5 to 11.0), whereas B exhibited the greatest activity at approximately *p*H 7.3.

Without a more exhaustive study of the properties of these three proteases, comparison with other similar enzymes from *B. subtilis* reported in the current literature may not be warranted. However, it might be noted that the enzymes designated "neutral" and "alkaline" in sporulation studies by Millet (14) appear similar to enzymes B and C of the present work, although no stimulation of "alkaline" protease by EDTA was reported by this author. More complete data on the extracellular enzymes, in which a third enzyme (presumably identical with our A) was isolated, was recently presented by Millet (15). On the other hand, the "basic" and "acidic" proteases studied by Boyer and Carlton (3), although similar in some other respects to enzymes A and C, differ in their reported heat stabilities and *p*H optima.

Since each of the three proteolytic enzymes A, B, and C separated on HA could be individually measured in a mixture containing all three by the methods already utilized in column chromatography, further identification studies were not required to proceed with the primary objective of this study—an analysis of the formation of these proteases during the process of sporulation.

Parameters of the sporulation system. The lactate medium employed permitted sporulation to occur approximately 16 hr after inoculation with heat-shocked spores. A typical sequence of events in this sporulation system is illustrated in Fig. 4A. The end of exponential growth is designated as t_0 (17). After inoculation at t_{-11} (that is, 11 hr prior to t_0) a germination lag of several hours occurred until growth on glucose commenced at t_{-9}. During this period the *p*H of the culture dropped from 7.6 to 5.2, following which the *p*H increased rapidly. Glucose was presumably exhausted in the medium by t_{-7} when another lag occurred during which adaptation to lactate took place. Final growth occurred on lactate between t_{-4} and t_0 but at a dramatically reduced rate (doubling time = 3 hr). The specific content of cellular dipicolinic acid increased beginning at about t_3 to t_4 almost coinciding with a rapid formation of refractile bodies which attained a maximal value of 80 to 90% by t_7. The curve for per cent heat-resistant spores paralleled refractile body formation but lagged approximately 30 min behind it, beginning at $t_{4.5}$ to t_5. Since chains of cells of various lengths were observed throughout growth, determination of viable cells and heat-resistant spores by the plating techniques utilized are only relative. They are included here for the purposes of comparison with other systems. At t_0 total viable cell concentration was approximately 2×10^8 per ml and at t_7 heat-resistant spores constituted about 50 to 70% of the population.

The time sequences reported above were reproducibly obtained with variations in specific events generally no greater than 1 to 2 hr over the 18-hr time period of an experiment. The observations are in basic agreement with the sequence of events in sporulation found by other investigators (17).

TABLE 1. *Specificities of enzyme fractions*

Determination[a]	Activity of fractions		
	A	B	C
Substrate			
Azocasein	+[b]	+	−
BAEE	−	−	+
BTEE	−	−	+
ATEE	+	−	+
TAME	+	−	−
FAGLA	−	+	−
Gelatin	+	+	+
p-Nitrophenylacetate	−	−	+
Inhibitor[c]			
EDTA	−[d]	+	−
PMSF	+	−	+
Cysteine	+	+	+

[a] Abbreviations: BAEE, benzoylarginine ethyl ester; BTEE, benzoyltyrosine ethyl ester; ATEE, acetyltyrosine ethyl ester; TAME, tosyl-L-arginine methyl ester; FAGLA, furylacryloyl-glycyl-L-leucine amide.

[b] Symbols for substrate: +, active; −, inactive.

[c] Abbreviations: EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluroide.

[d] Symbols for inhibitor: +, inhibited; −, not inhibited.

Intracellular activities of sporulating hpr-97. As Table 1 indicates, substrates are available which are relatively specific for each of the three proteases isolated above [i.e., TAME (tosyl-L-arginine methyl ester, N B Co.), FAGLA (furylacryloyl-glycyl-L-leucine amide, Cyclo), and BAEE or BTEE for A, B, and C, respectively]. Assays involving the use of these compounds, however, require spectrophotometric detection of hydrolysis at wavelengths in the UV region. The extremely high UV absorption contributed by the amount of crude cell extract required for accurate determinations precluded the use of UV-absorbing substrates for assays involving intracellular activities. The activity of A and B, therefore, was measured by hydrolysis of azocasein (with or without EDTA), yielding products which could be followed spectrophotometrically in the visible light region (430 nm). The same considerations dictated the use of *p*-nitrophenylacetate as a substrate for enzyme C. Although BAEE activity (specific for C) as measured in

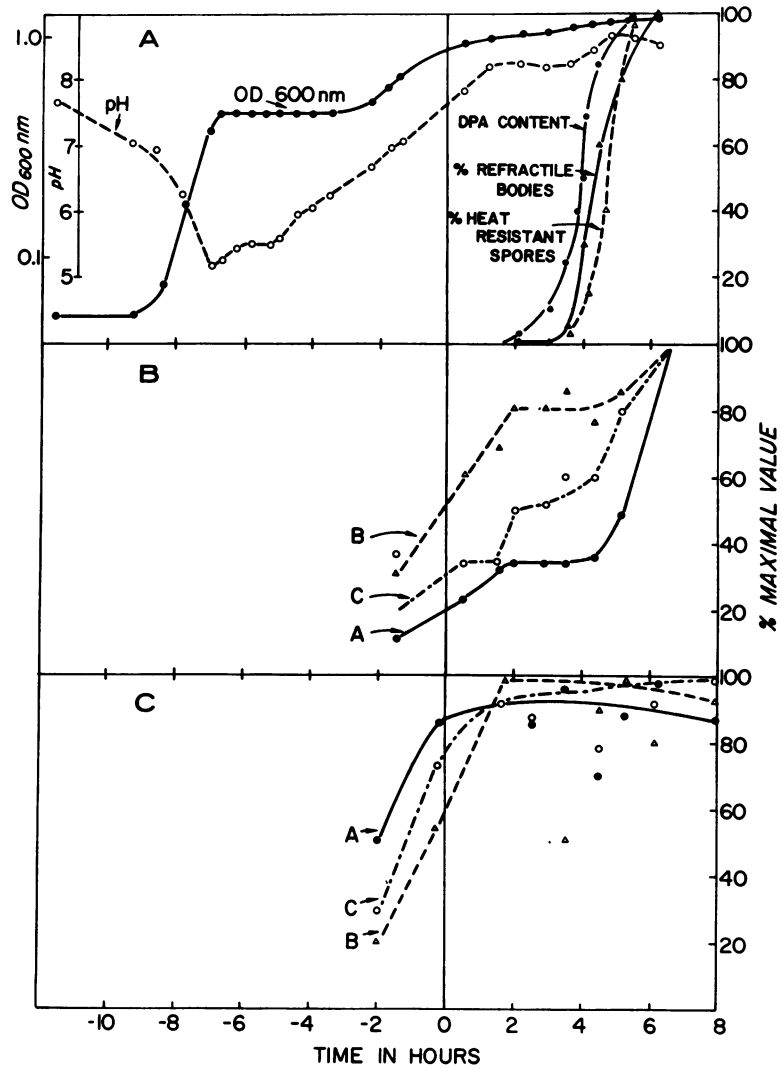

FIG. 4. *Time course of sporulation and protease activities. The pH values and optical density readings at 600 nm were made on samples removed at intervals from a fermentor culture of hpr-97. These are plotted in 4A on a linear scale versus time. Values for content of dipicolinic acid (DPA), refractile body formation, and heat-resistant spores are presented in 4A as per cent maximum value attained. In Fig. 4B (intracellular) and 4C (extracellular) specific activity values of the three proteases are plotted as per cent maximal specific activity versus sampling time. Assays for activity are described in Materials and Methods.*

cell extracts by the difference in slope plus or minus substrate closely coincided with *p*-nitrophenylacetate hydrolysis, the latter compound could be hydrolyzed by other esterases which might be present. Determinations of activity of enzyme C with this substrate therefore must be accepted with caution.

Assays for intracellular activities were made on cell extracts after breakage of cell suspensions by sonic treatment, centrifugation to remove debris, and dialysis. The results given in Fig. 4B are plotted as per cent maximal specific activity versus time of sampling. Results from some samples taken during the lag prior to growth on lactate indicated that specific activities of all three enzymes were either constant or decreasing slightly during this time. Generally, sampling was begun at the time when growth on lactate commenced. Rapidly increasing rates of synthesis of the three proteases occurred throughout this growth period. The rates of synthesis became constant at about t_2, and specific activities remained relatively unchanged for several hours until at about t_4 a "burst" of synthesis occurred.

The most dramatic increases in specific activity at this time were associated with activity A. As comparison with Fig. 4A shows, the final increased rates of protease formation occurred in the same general time period as spore-specific events such as dipicolinic acid synthesis, formation of refractile bodies, and production of heat-resistant spores. Proteolytic activities appeared to reach a maximum at the time coinciding most closely with appearance of heat-resistant spores.

In a single experiment, enzymes A and B appeared to follow the same general pattern in strain 168 as that given above for hpr-97 (enzyme C was not followed).

A comparison of specific activity data of enzymes A and B at comparable times in strains 168 and hpr-97 is given in Table 2. It is apparent that strain hpr-97 can be classified as an overproducer of enzyme B since the specific activity of this enzyme is approximately 9 to 10 times higher in hpr-97 throughout the course of sporulation. Initially, enzyme A also is present in hpr-97 at a specific activity about three times that found in strain 168. However, this value changes with time so that by t_6 both strains have values which are almost identical. A common feature is shared in both strains, the gradually decreasing ratio of B/A specific activities as sporulation progresses. This may prove to be an occurrence more typical of sporulation than are levels of individual proteases.

Extracellular activities of sporulating hpr-97. Results from analysis of either $(NH_4)_2SO_4$ precipitated or freeze-thaw concentrated supernatant fluid indicate that extracellular specific activities of all three proteases reached a maximum value about t_1 or t_2 and remained relatively constant thereafter (Fig. 4C) in agreement with previous investigations (14, 22).

Inspection of the specific activities of A and B found in supernatant fluid of strains 168 and hpr-97 (Table 3) indicates that the differences demonstrated intracellularly were reflected in products found in the medium. Mandelstam and Waites (13) also found wild-type *B. subtilis* 168 and a neutral protease-negative mutant, E22, had extracellular proteolytic activity proportional to the intracellular content. Supernatant fluid of hpr-97 contained approximately twice the specific activity of A and about 10 times the specific activity of B as is found in supernatant fluid from strain 168. Since the specific activities of A and B in the medium were about 500 times higher than those found intracellularly, it was concluded that these proteolytic activities result from excretion rather than cell lysis. Excretion may be purely a function of intracellular concentration at a particular time. Although such excretion phenomena seem to have a more complex basis in vegetative cells, this may not be the case during sporulation. Bernlohr (2) has found permeability changes during sporulation of several *Bacillus* strains (including *B. subtilis* 23) such that intracellular amino acid pools show a transient minimum at about t_0 (and a maximum at about t_5). This is the time period at which extracellular proteolytic activities also reach maximum values.

DISCUSSION

Three proteases have been demonstrated in both cells and supernatant medium from sporulating cultures of strain 168 and hpr-97, a mutant over-producing neutral protease. None of these activities appears to be "spore-specific" in the sense that they are produced only during sporulation, since the data given indicate very rapid synthesis takes place in cells in a glucose-free medium during the growth period prior to t_0 (defined here as the time cell division ceases). Din and Chaloupka (4) have shown that casein hydrolysate in the absence of glucose stimulates protease formation in cells of *B. megaterium* KM in the stationary phase of growth, but it is not known whether the same effect occurs during the exponential phase of growth on carbon sources other than glucose. It appears that cessation of protease synthesis shortly after t_1 must be attributable to some controls other than those affected by the presence or absence of glucose,

TABLE 2. *Comparison of intracellular protease activities on azocasein*[a]

Enzyme	Specific activity of								
	Strain hpr-97			Strain 168			Ratio of strain hpr-97–168		
	t_0	$t_{1.75}$	t_6	t_1	$t_{1.5}$	t_6	t_1	$t_{1.5}$	t_6
A	0.0148	0.0203	0.0603	0.00468	0.010	0.053	3.17	2.03	1.13
B	0.0596	0.098	0.0957	0.00603	0.0084	0.0105	9.90	9.30	9.13
Ratio (B/A)	4.03	3.84	1.59	1.29	0.84	0.20			

[a]Azocasein units are defined as the change in absorbancy at 430 nm per 60 min per ml under the conditions described in Materials and Methods.

TABLE 3. *Comparison of extracellular protease activities on azocasein*[a]

Enzyme	Specific activity of					
	Strain hpr-97		Strain 168		Ratio of strain hpr-97 168	
	$t_{2.5}$	t_6	t_3	t_6	t_3	t_6
A	9.40	11.30	4.34	5.07	2.17	2.23
B	38.7	44.0	4.20	4.13	9.23	10.65
Ratio (B/A)	4.12	3.89	0.97	0.82		

[a] Azocasein units are defined as the change in absorbancy at 430 nm per 60 min per ml under the conditions described in Materials and Methods.

this carbon source having been exhausted about 7 hr previously. In any case, a second round of intracellular protease synthesis occurring at about t_4 can be regarded as a sporulation-associated event. It is evident that the control of enzymes B and A occurs by different mechanisms since plots of specific activity data from a number of experiments indicate that ratios of B/A in cells of both strains 168 and hpr-97 change in an apparently nonlinear fashion during the course of sporulation.

The fact that the specific activity ratio of B/A was found to be maximal at early times in sporulation of both 168 and hpr-97 strains provides some support for the conclusion of Mandelstam and Waites (13) that neutral protease (which most closely resembles enzyme B in its properties) is responsible for the early protein turnover (between t_0 and t_5) which occurs in sporulating cells. Data provided in this report show that the increases in specific activity of all three enzymes at t_4 are most pronounced in the case of enzyme A. Accordingly, enzyme A may be involved in this later period of sporulation. Times of maximal protease production correspond notably to the times reported by Spudich and Kornberg (20) as those in which "core" and spore "coat" proteins are synthesized. Their data show core protein is formed at early times when protein turnover attributed to neutral protease (or enzyme B) is occurring. Two kinds of spore "coat" protein were made several hours later, overlapping in time the period in which refractile bodies are observed and heat-resistant spores appear. This is the period in which both proteases A and C exhibit major specific activity increases. It is tempting to speculate that these enzymes may have a role in supplying the specific peptides required for spore coats.

Mandelstam and Waites (13) have shown that an Sp^- mutant (EF1) of *B. subtilis* Marburg 168 which lacks neutral protease does not exhibit the early protein turnover found in the wild type during sporulation. However, mutant 3F1 does show a later (t_6) breakdown of protein which these authors attribute to the subsequent appearance of an alkaline protease. Enzyme A was found in the present report to appear at approximately this time and may be the enzyme ascribed to 3F1. If so, it can be concluded that the presence of neutral protease (enzyme B, lacking in 3F1) is not a prerequisite for formation of alkaline protease (enzyme A). Among the Sp^- mutants isolated in this laboratory is one in which the reverse situation occurs, namely, production of neutral protease (enzyme B) but not alkaline protease (enzyme A), indicating that presence of A is not required for synthesis of B. Consequently, sequential reading of the chromosome does not appear to be involved in protease production during sporulation.

Antigenic studies by Waites (21) have shown that at least five of the seven antigens detected by antiserum to spores are also present in vegetative cells although low concentrations of several of these suggest a basal level. In our experiments detectable levels of three proteases are found in vegetative cells during growth on lactate. These are therefore presumably present at some basal level even in growth medium containing glucose. The fact that two of these proteases (A and B) are relatively heat labile, as is one of the antigens detected by spore antiserum in Waites' experiments, may be more than coincidental.

In conclusion then, it can be stated that, although the precise role of proteases during sporulation remains uncertain, evidence for their formation at specific times during the process is supported by the present study.

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