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Bacillus megaterium Spore Protease: Purification, Radioimmunoassay, and Analysis of Antigen Level and Localization During Growth, Sporulation, and Spore Germination

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The protease which initiates the massive protein degradation early in bacterial spore germination has been purified from *Bacillus megaterium* spores. The enzyme has a molecular weight of 160,000 and contains four apparently identical subunits, but only the tetramer is enzymatically active. A radioimmunoassay has been developed for this enzyme and has been used to show that the protease is absent from growing cells, but appears early in sporulation within the developing forespore. In contrast, the protease antigen disappears rapidly during spore germination, in parallel with the loss in enzyme activity.

Dormant spores of various *Bacillus* species carry out no detectable endogenous metabolism or macromolecular biosynthesis (3, 11). However, during the first minutes of spore germination metabolism and RNA and protein synthesis begin (11). Protein synthesis early in the germination of *Bacillus megaterium* spores is dependent on endogenous amino acid reserves which are generated by degradation of dormant spore protein (17). Approximately 20% of the protein of spores of various *Bacillus* species is degraded in the first minutes of germination; the major proteins degraded are a group of low-molecular-weight proteins (termed A, B, and C proteins in *B. megaterium*) which are unique to the spore stage of the life cycle (14).

In *B. megaterium* the degradation of the A, B, and C proteins is initiated by a specific endoprotease (10, 15). This enzyme is active only on the A, B, and C proteins, in which it makes a few endoproteolytic cleavages (15). Although this protease acts rapidly on its substrate proteins in the first minutes of spore germination, there is no such interaction within the developing or dormant spore, despite the presence of both enzyme and substrate (10, 19). Consequently, the protease must be regulated in some fashion such that its activity is low or absent in the developing and dormant spore yet increases rapidly early in spore germination.

Unfortunately, there have been two significant barriers to detailed studies of the regulation of this spore protease in vivo. (i) The assay of the spore protease in vitro (by measurement of degradation of a crude mixture of the A, B, and C proteins) is not specific for this protease. In particular, quantitation of the spore protease in sporulating cells, which contain extremely high levels of nonspecific proteases, is impossible without partial purification (10). (ii) At present there is no method for detecting an inactive form(s) of the protease such as a zymogen—a common mechanism for regulating the enzymatic activity of proteases in vivo. One assay which could surmount both of these barriers is a radioimmunoassay. Consequently, we undertook the purification of the protease from *B. megaterium* spores in order to (i) determine key physical and chemical properties of the enzyme, (ii) develop a radioimmunoassay for the enzyme, and (iii) use this radioimmunoassay to study the distribution of the protease antigen in various stages of growth, sporulation, and spore germination and in the two compartments of the sporulating cell.

MATERIALS AND METHODS

Bacteria, spores, and reagents. All work was carried out with *B. megaterium* QM B1551 (originally obtained from H. S. Levinson, U.S. Army Natick Laboratories, Natick, Mass.). Cells were grown and sporulated at 30°C in supplemented nutrient broth, and spores were harvested, washed, and stored as previously described (16). ^{125}I-labeled Bolton-Hunter reagent (2,000 mCi/μmol) (1) was obtained from New England Nuclear Corp. Goat anti-rabbit immunoglobulin G (IgG) serum was obtained from Miles Laboratories and was fractionated and stored as previously described (13). The crude mixture of proteins A, B, and C from *B. megaterium* spores as well as partially purified aminopeptidase were also prepared as previously described (15).

Purification of protease. The initial steps in purification of the spore protease were carried out on spores from an 11-liter culture which yielded ~8 g (dry

weight) of spores; the cleaned spores were first germinated and then extracted. Spores were suspended in water at 25 mg (dry weight) per ml, heat shocked (10 min, 60°C), and cooled in ice. The spores were germinated at 30°C with 2.5 mg of spores in 1 ml of 50 mM Tris-hydrochloride (pH 7.4)–100 mM glucose–10 mM KCN. The KCN was added to prevent the energy-dependent loss in spore protease during germination (15). The initiation of spore germination was complete in 10 min, and the suspension was chilled by the addition of ice and centrifuged (15,000 × g, 10 min). The pellet was suspended at a concentration of 30 mg of the original spores in 1 ml of 100 mM Tris-hydrochloride (pH 8.7 at 37°C)–5 mM $CaCl_2$ at 37°C. Lysozyme (1.5 mg/100 mg of spores) was added, and after 10 min at 37°C the suspension was chilled and centrifuged (15,000 × g, 10 min), and the pellet fraction was discarded. All subsequent steps were carried out at 4°C. Protein was precipitated by the slow addition of 390 g of ammonium sulfate per liter of supernatant fluid, the suspension was centrifuged (10 min, 12,000 × g), and the supernatant fluid was discarded. The precipitated protein was dissolved in 25 ml of 10 mM Tris-hydrochloride (pH 7.4 at 4°C)–5 mM $CaCl_2$ and dialyzed against 1 liter of the same buffer containing 20% glycerol with one change of buffer. The dialyzed sample was centrifuged (10 min, 10,000 × g), and to the supernatant fluid [$(NH_4)_2SO_4$ precipitate fraction] was added 8 ml of a streptomycin sulfate solution (100 mg/ml in 10 mM Tris-hydrochloride–5 mM $CaCl_2$–20% glycerol with the pH adjusted to 7.4). After centrifugation (10 min, 10,000 × g) the supernatant fluid was frozen and stored at −80°C. This step gave little purification, but did remove nucleic acid which would otherwise have interfered with subsequent chromatography on DEAE-Sephadex. When the equivalent of 40 g (dry weight) of spores had been treated in this manner, the separate supernatant fluids were thawed, pooled, and adjusted to 80 mM NaCl and 40 mM Tris-hydrochloride (pH 7.4 at 4°C). This material was applied to a column (5 by 20 cm) of DEAE-Sephadex equilibrated in 100 mM NaCl–50 mM Tris-hydrochloride (pH 7.4 at 4°C)–5 mM $CaCl_2$–20% glycerol (buffer A). The column was washed with 800 ml of buffer A, and the enzyme was eluted with a linear gradient of 0.1 to 0.4 M NaCl in buffer A. Two liters of each solution was used, and fractions of 20 ml were collected. Fractions with high protease activity were pooled, dialyzed against 10 mM Tris-hydrochloride (pH 6.8 at 4°C)–5 mM $CaCl_2$–20% glycerol (buffer B) for 3 h and applied to a DEAE-cellulose column (16 by 2.3 cm) equilibrated in buffer B. The pH of buffer B was adjusted to 6.8 (at 4°C); this value was crucial for good enzyme purification (see below). Enzyme was eluted with a linear gradient of 75 to 250 mM NaCl in buffer B by using 400 ml of each solution and collecting 8-ml fractions. Active fractions were pooled and adjusted to the same pH and conductivity as buffer C (50 mM Tris-hydrochloride [pH 8.5]–100 mM NaCl–5 mM $CaCl_2$–20% glycerol). The enzyme was then concentrated by adsorption to a small (1-ml) DEAE-Sephadex column equilibrated in buffer C, followed by elution with 0.4 M NaCl in buffer C. Storage was at −80°C in 100-μl samples.

Raising of protease antibody. Initial attempts to raise antisera against purified native protease were unsuccessful. Consequently we used as an antigen protease which was cross-linked to itself with glutaraldehyde (13). A 0.4-ml amount of purified protease (700 μg/ml) was dialyzed overnight at 4°C against 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.4)–20% glycerol–5 mM $CaCl_2$ (buffer D). The dialyzed protein was then mixed with an equal volume of 14 mM glutaraldehyde in buffer D, and the mixture was incubated at 24°C. After 2 h, 1.2 ml of 50 mM lysine was added to destroy remaining glutaraldehyde, and the cross-linked protein was dialyzed overnight against several changes of 1 liter of 1 mM Tris-hydrochloride (pH 7.0) and lyophilized. Before use, the cross-linked antigen was dissolved in 1 ml of 10 mM Tris-hydrochloride (pH 7.5) and mixed with 1 ml of Freund complete adjuvant. This mixture was injected intradermally in five sites on the back of a New Zealand white female rabbit (2.7 kg). Five weeks later the rabbit was injected intravenously with an additional 0.5 mg of cross-linked protease in 10 mM Tris-hydrochloride (pH 7.5), the rabbit was bled 6, 8, and 10 days after the injection. The boosting and bleeding procedure was repeated 5 weeks later, but sera from all bleedings gave similar titers. Sera were allowed to clot overnight at 4°C and centrifuged (30 min; 25,000 × g), and the γ-globulin fraction was isolated and stored as described previously (13). The source of control γ-globulin was also as previously described (13).

Iodination of purified protease. Initial attempts to iodinate the protease by the chloramine T procedure gave very little incorporation of label into the enzyme; consequently we turned to the Bolton-Hunter reagent (1). Purified protease was dialyzed against buffer D at pH 8.0, and 25 μg in 50 μl was added to 1 mCi of dry Bolton-Hunter reagent. The mixture was incubated for 1 h at 0°C and then 3 h at 4°C, followed by the addition

TABLE 1. Purification of spore protease[a]

Step	State of purification	Total units	Sp act (U/mg of protein)[b]	Recovery (%)	Purification (fold)
1	Dormant spore[c]	4×10^5	24	100	—
2	Ammonium sulfate precipitate	4×10^5	6×10^2	100	25
3	Streptomycin sulfate supernatant fraction	3.6×10^5	5×10^2	90	21
4	DEAE-Sephadex pool	1.5×10^5	3.2×10^3	38	133
5	DEAE-Cellulose pool	4.6×10^4	5×10^4	12	2,080

[a] The protease was purified from 40 gm of dry spores as described in the text.
[b] Protein was determined by the procedure of Lowry et al. (8).
[c] Values were determined for dormant spores as described previously (15).

of 500 μl of 0.2 M glycine in buffer D at pH 8.1. After 5 min at 4°C the mixture was applied to a column of Sephadex G-25 (1 by 12 cm) equilibrated at room temperature with buffer D at pH 8.0 plus 0.25% gelatin and eluted with this buffer. Iodinated protease fractions were detected with a Geiger counter, pooled, dialyzed at 4°C against two changes of 500 ml of buffer A, and stored at -80°C in small aliquots. The yield of iodinated protease was 50 to 100 μCi, and approximately 85% of the label comigrated with the purified protease on acrylamide gel electrophoresis run by the method of Laemmli (6). Approximately 10% of the counts ran with the dye front, and this percentage increased upon prolonged (several months) storage of the iodinated protease.

Radioimmunoassay procedure. Radioimmunoassays were carried out in 250 μl of RIA buffer (0.1 M NaCl-25 mM KPO_4 [pH 7.4]-2 mg of bovine serum albumen per ml; use of Tris-hydrochloride buffers with or without glycerol or $CaCl_2$ gave similar results). Each reaction also contained ~2 ng of iodinated protease (~10^4 cpm) and 0.3 μl of control γ-globulin; the amount of immune γ-globulin routinely added was also 0.3 μl. This amount of antiserum precipitated 60 to 65% of the labeled antigen in the reaction. For some experiments—in particular those where sporulating cell extracts were to be assayed—the RIA buffer also contained 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride. These additions had no affect on the reactivity of either purified protease or germinated spore extracts in the radioimmunoassay. However, their addition to assays of sporulating cell extracts was necessary to prevent digestion of the iodinated antigen by the high level of nonspecific proteases in these extracts.

Reaction mixtures containing control γ-globulin, immune γ-globulin, [^{125}I]protease, and samples to be tested for protease antigen were incubated for 1 h at 37°C and then overnight at 4°C. Antibody-antigen complexes were precipitated by the addition of goat anti-rabbit IgG as described previously (13), and the pellet fraction was counted in a γ-counter. Determination of unknown amounts of protease antigen was by reference to a calibration curve constructed by using the purified protease. The antigenic unit was defined as the ratio of the amount of protease antigen in an extract divided by the amount of protease antigen in a lysozyme extract of germinated spores (standard extract) prepared as described above, when extracts from equivalent amounts of cells or spores (or both) were assayed. Relative specific antigenic units were defined as the amount of protease antigen in an extract per milligram of protein divided by the amount of protease antigen in our standard extract per milligram of protein in the standard extract.

The specificity of the radioimmunoassay for the spore protease was established by the following criteria. (i) Control reactions lacking immune serum precipitated only 10 to 15% of the [^{125}I]protease, and most (~70%) of the counts in the precipitate ran with the dye front on 10% acrylamide gels run by the method of Laemmli (6). (ii) In immune precipitates approximately 85% of the label comigrated with the purified protease (the majority of the remainder was at the dye front) in 10% acrylamide gels run by the method of Laemmli (6), and the addition of enough purified protease to remove approximately half of the counts from the immune precipitate removed them only from the position of the purified protease on gel electrophoresis. (iii) Electrophoretic analysis on 10% acrylamide gels of material in the supernatant fraction of radioimmunoassays of spore or sporulating cell extracts showed that the material not precipitated had the same molecular weight as that of the starting iodinated protease.

Extraction of spores, sporulating cells, or germinated

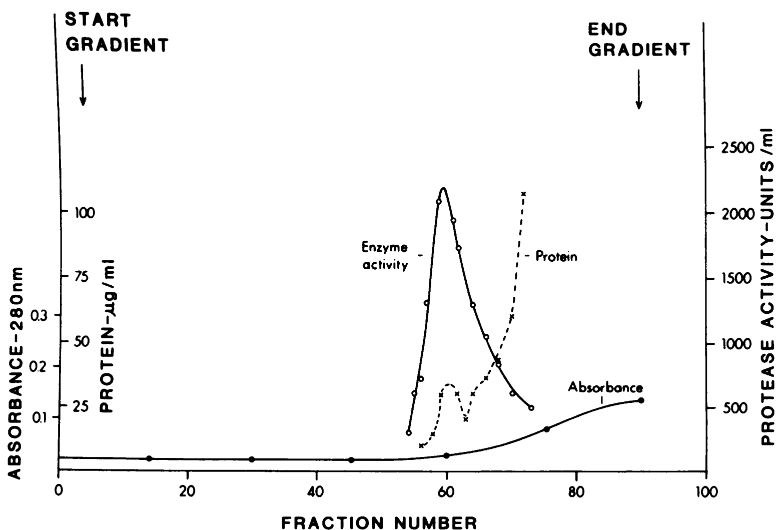

FIG. 1. DEAE-cellulose chromatography of spore protease. Protease was purified from 40 g of spores through the DEAE-Sephadex step and then run on a DEAE-cellulose column as described in the text. Protein was determined by the method of Bradford (2).

spores. Cells were grown in supplemented nutrient broth, and spores were germinated as described above, but at 2.8 mg/ml. Samples (50 ml) of these cultures were centrifuged (10 min; 10,000 × g), and the pellet fraction was stored frozen. Small samples (5 ml) were also harvested as described above, the pellet fraction was extracted for 10 min with 1 ml of boiling water and centrifuged (10 min; 8,000 × g), and the supernatant fluid was analyzed for dipicolinic acid.

Protease antigen was extracted from the frozen cell or spore pellets by sonication. The samples were suspended in 3 ml of 100 mM Tris-hydrochloride (pH 7.5 at 5°C)–1 mM EDTA–0.1 mM phenylmethylsulfonyl fluoride–20% glycerol and were disrupted by sonication in the presence of glass beads (400 mg). Control experiments showed that this procedure caused no loss in the antigenicity of purified spore protease, nor did it allow loss in the antigenicity of purified protease added to sporulating cells. When the EDTA and phenylmethylsulfonyl fluoride were omitted from the sonication buffer, added spore protease antigen was destroyed in the sporulating cell extracts, presumably by digestion by the high level of nonspecific proteases present.

Fractionation of sporulating cells into mother cell and forespore compartments. Sporulating cells (100 ml) were harvested and converted to protoplasts and washed, and forespores were isolated as previously described (19). These samples were broken by sonication as described above. In one case the sonication of protoplasts was carried out initially for only 15 s; this sample was then centrifuged (5 min; 10,000 × g) to yield a supernatant fraction (mother cell cytoplasm) and a pellet fraction (forespores and a few unbroken protoplasts). The supernatant fraction was immediately made 0.1 mM in phenylmethylsulfonyl fluoride and 12 mM in EDTA and frozen. Forespores were then purified from the pellet fraction and completely disrupted as described above.

Glycerol gradient centrifugation. Glycerol gradient centrifugation to separate the protease tetramers from monomers was carried out at 5°C in an SW 50.1 rotor for 20 or 75 h at 35,000 rpm. Centrifugation was in linear 12.5 to 30% glycerol gradients in 50 mM Tris-hydrochloride (pH 7.5 at 5°C) with either 5 mM $CaCl_2$ or 10 mM EDTA. Purified protease (12 μg) was applied to each gradient with markers of pig heart malate dehydrogenase (20 μg) and beef heart lactate dehydrogenase (10 μg).

Analytical procedures. The protease was assayed as described previously (15) by its ability to make endoproteolytic breaks in a crude mixture of the A, B, and C proteins. The presence of these endoproteolytic breaks was detected by aminopeptidase digestion followed by reaction with ninhydrin as described previously (assay procedure 3 [15]). One unit of enzyme is defined as a change in absorbance of 1.0 per 30 min under standard assay conditions (15). Protein was determined either by the procedure of Lowry et al (8), or in one case as noted by the procedure of Bradford (2). The molecular weight of the denatured protease was determined by sodium dodecyl sulfate-acrylamide gel electrophoresis (20) with markers of myoglobin, chymotrypsinogen, ovalbumin, and serum albumin. The Stokes radius of the native enzyme was determined by gel filtration on Sephadex G-200 in buffer A with markers of catalase, glutathione reductase, and lactic dehydrogenase (7). The sedimentation coefficient of the native enzyme was determined by centrifugation in a glycerol gradient (15 to 40%) containing 0.1 M NaCl, 50 mM Tris-hydrochloride (pH 7.4), and 5 mM $CaCl_2$ with markers of catalase, alcohol dehydrogenase and β-galactosidase (9). The molecular weight of the native enzyme was obtained from a straight-line plot of molecular weight versus ($a \times S$) (where a is the Stokes radius [$\times 10^{-1}$ nm] and S is $s_{20,w} \times 10^{13}$) generated by using data in the literature (18). Chemical cross-linking of native enzyme by the method of Davies and Stark (4) was carried out at a protein concentration of 0.3 mg/ml. The products were analyzed by sodium dodecyl sulfate-acrylamide gel electrophoresis (20), and their mobilities were compared

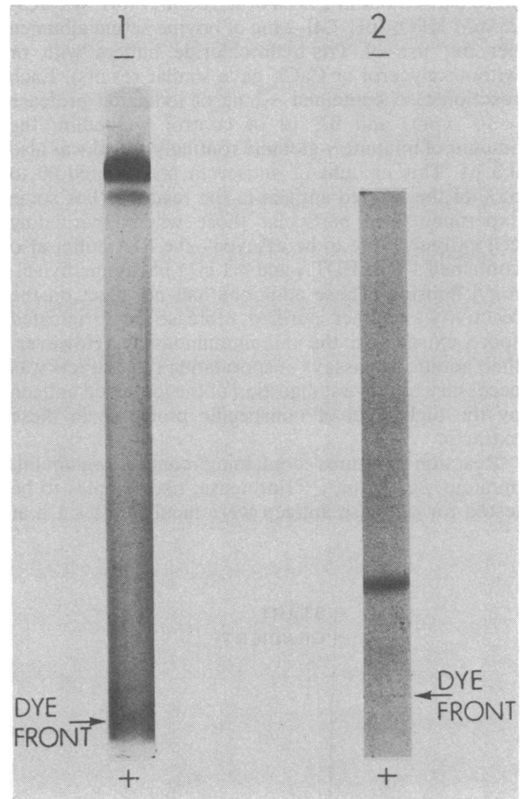

FIG. 2. Gel electrophoresis of purified spore protease under nondenaturing (1) or denaturing (2) conditions. 1, Protease (20 μg) from step 5 (Table 1) was applied to a 7.5% acrylamide gel that was 50 mM in Tris-acetate (pH 7.5 at 20°C) as well as 5 mM in $CaCl_2$ and 20% in glycerol to preserve the protease activity during electrophoresis. Two identical gels were run; one was stained with Coomassie blue (shown in the figure), and the other was cut into 0.5-cm slices from which the enzyme was extracted and assayed for protease (not shown). Approximately 20% of the enzyme activity applied to the gel was recovered. 2, Sodium dodecyl sulfate-acrylamide gel electrophoresis of 8 μg of spore protease from step 5 (Table 1) was carried out with 7.5% gels as described by Laemmli (6).

with those of β-galactosidase, phosphorylase b, serum albumin, and ovalbumin.

RESULTS

Purification of the spore protease. The procedure for protease purification was a modification of an earlier procedure which gave a partial purification (15) (Table 1). The modifications of the original procedure were: (i) scaling up the procedure to use 40 g of spores; (ii) use of streptomycin sulfate for nucleic acid removal; (iii) an ammonium sulfate fractionation step; and (iv) modification of the salt gradient on the DEAE-cellulose column. In the final step of the purification procedure the enzyme eluted as a single peak of activity from the DEAE-cellulose column, although the peak was significantly skewed (see below), and the enzyme activity coeluted with a small protein peak preceding the great majority of the protein (Fig. 1). For good purification in this final step it was essential to run the DEAE-cellulose column at pH 6.8; at a higher pH (8.0) the enzyme activity ran with the majority of the protein, and the activity peak was even more skewed than at pH 6.8 (data not shown). However, even upon chromatography at pH 6.8 the enzyme exhibited somewhat anomalous behavior as the activity peak trailed off significantly (Fig. 1). Rechromatography of the leading edge of the enzyme activity peak from DEAE-cellulose also resulted in an activity peak which trailed off badly (data not shown).

The modified purification procedure resulted in a 2,000-fold purification of the enzyme with a 12% yield. The yield was low in part because only narrow cuts of enzyme activity were pooled from the column steps. Electrophoresis of enzyme from step 5 under nondenaturing conditions gave a single major band comprising at least 90% of the protein and a single minor band (Fig. 2). Electrophoresis on a nondenaturing gel for five times longer than for the gel shown in the figure also gave only a single major and a single minor band, but these were now separated by 1 cm (data not shown). Extraction and assay of enzyme from either of these gels showed that protease activity was associated only with the major protein band (data not shown). Analysis of the most purified protease fractions by electrophoresis on a denaturing gel again gave only a single major band and no obvious minor bands (Fig. 2). Possibly the minor band seen on the nondenaturing gel was denatured (possibly monomeric; see below) protease. Although the most purified protease preparations may not be homogeneous, it appears certain that >90% of the protein in these preparations is the spore protease.

Physical properties of the purified enzyme. The purified native enzyme had a Stokes radius of 5.2 nm as determined by gel filtration on Sephadex G-200 as described above. The sedimentation coefficient ($s_{20,w}$) of the native enzyme was 7S (data not shown; see above). These two values were used to calculate a native molecular weight for the spore protease of 160,000 by the method of Siegel and Monty (18). In contrast, the molecular weight of the denatured protease was 40,000 as determined by sodium dodecyl sulfate-gel electrophoresis by the method of Weber and Osborn (20). These two values strongly suggested that the spore protease was a tetramer of identical subunits. The tetrameric structure of the protease was confirmed by covalent cross-linking of the native enzyme, followed by analysis of the products on sodium dodecyl sulfate-acrylamide gels as described by Davies and Stark (4). This procedure generated

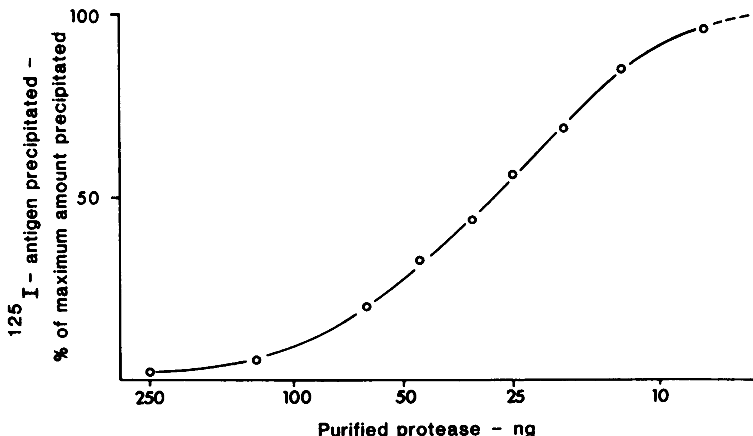

FIG. 3. Calibration curve for radioimmunoassay. The radioimmunoassay was carried out as described in the text with various amounts of purified spore protease.

new protein bands of molecular weight 155,000 (tetramer) and 85,000 (dimer) (data not shown). Although little, if any, trimer was produced, the absence of species larger than tetramer indicated again that the native enzyme is a tetramer and is composed of identical subunits.

Use of radioimmunoassay to determine the activity of protease monomers. With sufficient amounts of purified spore protease available, we were able to raise an antibody to the protease and set up a radioimmunoassay which could easily detect 15 ng of the enzyme (Fig. 3). Radioimmunoassay of serial dilutions of either purified protease or a lysozyme extract of germinated spores generated curves with identical slopes (data not shown), suggesting that the spore protease is the major reactive antigen in spore extracts. Assays of spore extract gave a level for the protease of 300 μg/g of dry spores (data not shown). The value calculated from the data in Table 1 is somewhat lower (190 μg/g of dry spores), suggesting that some of the enzyme may have been denatured or otherwise altered during purification.

The reactivity in the radioimmunoassay of the purified spore protease or the enzyme in spore extracts was not affected when protease activity was first abolished by treatment with phenylmethylsulfonyl fluoride or EDTA (or both). This finding suggested that the radioimmunoassay could be used to determine whether the protease monomers were inactive. Sedimentation of purified enzyme on glycerol gradients with $CaCl_2$ for a relatively short time gave a major peak of protease antigen sedimenting at 7S (tetramer) with a zone of antigen toward the top of the gradient (presumably monomer) which was smaller than the malic dehydrogenase marker. Protease activity was associated only with the 7S peak (Fig. 4A). Sedimentation of enzyme on glycerol gradients with $CaCl_2$ for a longer time moved the tetramers to the bottom of the tube and resolved a minor antigen peak which sedimented more slowly than malic dehydrogenase (molecular weight, 67,000) (Fig. 4B). However, protease activity was associated only with the antigen at the bottom of the tube (presumably tetramer). When protease was sedimented for a

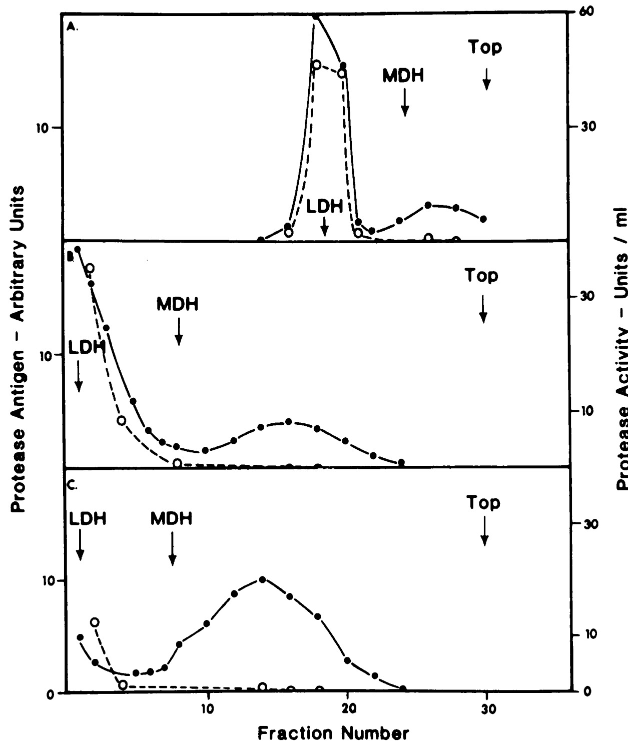

FIG. 4. Glycerol gradient centrifugation of purified spore protease (A) with $CaCl_2$ for 20 h, (B) with $CaCl_2$ for 75 h, or (C) with EDTA for 75 h. Glycerol gradient centrifugation was carried out as described in the text, and gradient fractions were analyzed for protease activity and antigen. In this experiment the amount of protease antigen in the tube with the most antigen was arbitrarily given a value of 20. The arrows labeled LDH and MDH give the position of the peak tubes of the lactate dehydrogenase (molecular weight, 140,000) and malic dehydrogenase (molecular weight, 67,000) markers, respectively.

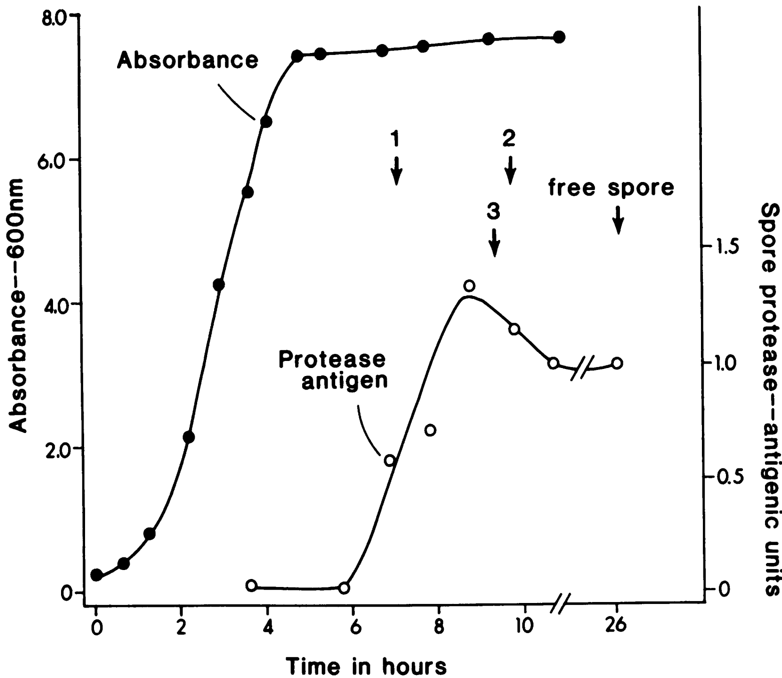

FIG. 5. Level of protease antigen during growth and sporulation. Cells were grown, harvested, broken, and analyzed for protease antigen or dipicolinic acid as described in the text. Arrows 1 and 2 denote the times samples were taken for analysis of forespore and protoplast fractions. Arrow 3 denotes the time when 50% of the maximum dipicolinic was accumulated.

long time with EDTA in the gradient, the peak of antigen sedimenting as a monomer was greatly increased, whereas the amount of antigen at the bottom of the tube (tetramer) was decreased; again, enzyme activity was found only at the bottom of the tube (Fig. 4C). These data suggest (i) that the tetramer is the active form of this enzyme and (ii) that the function of Ca^{2+} in the spore protease is not catalytic but structural, as was suggested previously on other grounds (15). That the subunits of the spore protease are inactive is also supported by the observation that dilute enzyme loses activity, but that the enzyme can be reactivated by reconcentration (15).

Use of radioimmunoassay to determine antigen level and location during growth, sporulation, and germination. The radioimmunoassay was also invaluable for determination of protease antigen levels in different stages of growth. Protease antigen was not detected in log phase or young sporulating cells and only appeared 1 to 2 h into stationary phase growth (Fig. 5). The time for accumulation of 50% of the maximum amount of protease antigen was approximately 2 to 3 h before accumulation of 50% of the maximum amount of dipicolinic acid (Fig. 5). Thus protease antigen accumulates at about the time of (or even slightly before) the accumulation of its substrates (14). After reaching its maximum, the level of protease antigen decreased slightly to the level found in the free spores (Fig. 5). Separation and assay of the mother cell and

TABLE 2. Distribution of protease antigen in the sporulating cell[a]

Time of harvest[b]	Relative specific antigenicity		
	Protoplast	Mother cell	Forespore
1	0.20[c]	—[d]	1.0[c]
2	—[d]	<0.07	1.0

[a] Samples were harvested, protoplasts were formed, and mother cell and forespore samples were isolated and analyzed as described in the text.

[b] Times of harvest refer to the numbered arrows in Fig. 5.

[c] Since the forespore at this stage of growth has approximately 20% of the total protoplast protein (19), these data indicate that >90% of the protease antigen of the protoplast is located in the forespore. This value is 1.0 even though only ~60% of the maximum amount of protease antigen has been accumulated, because forespore protein levels at harvest time 1 are significantly below those at harvest time 2 (19).

[d] —, Value not determined.

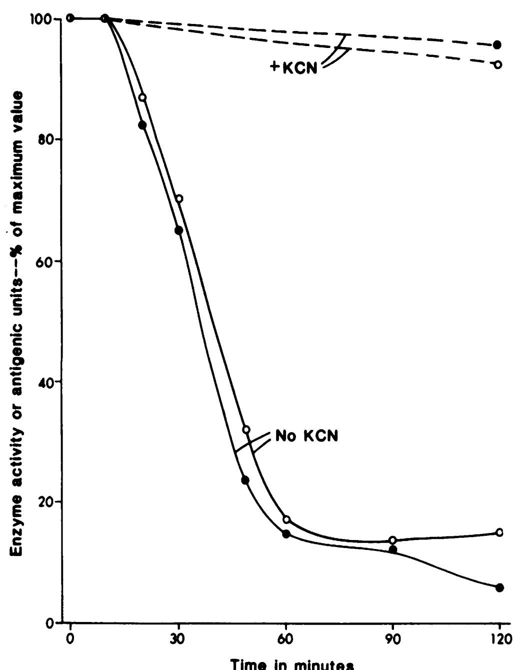

FIG. 6. Levels of protease antigen and activity during spore germination. Spores were germinated with or without KCN (10 mM), harvested, broken, and assayed for protease antigen and activity as described in the text. Symbols: (○) antigenic units; and (●) enzyme activity.

forespore compartments for protease antigen showed that at both times tested (Fig. 5) >90% of the protease antigen was in the forespore compartment (Table 2).

Previous work has shown that spore protease activity is lost during spore germination (15). The protease antigen was also lost during germination, in parallel with the loss of activity (Fig. 6). However, when the loss in protease activity during germination was prevented by germination with KCN (15), protease antigen was also not lost (Fig. 6). Previous work has demonstrated that the protease probably does not degrade itself in vitro (15). Thus it seems unlikely that it is involved in degrading itself in vivo.

DISCUSSION

Several of the properties of the *B. megaterium* spore protease determined in this work are unusual or abnormal. Thus the enzyme does not give a sharp peak of activity upon chromatography on DEAE-cellulose. Indeed, in previous work (10) we found that under some conditions two peaks of enzyme activity were obtained upon DEAE-cellulose chromatography. Since we could find no differences between these two peaks of enzyme activity in their catalytic activity or their electrophoretic mobility on native or denaturing gels (10; S. S. Dignam and P. Setlow, unpublished results), it is probable that the presence of these two peaks was a chromatographic artifact. The reason(s) for the abnormal chromatographic behavior of the spore protease is not clear. However, the anomolous behavior could be due to the dissociation of enzyme tetramers into monomers and their reassociation during chromatography if (i) the dissociation and reassociation steps were slow but significant under the conditions of chromatography, and (ii) monomers were retarded on DEAE-cellulose relative to tetramers.

The fact that the native enzyme contains subunits is of itself unusual in that most proteases described in the literature are monomeric with molecular weights of 20,000 to 60,000. However, there are some exceptions to this rule, one being clostridial collagenase, which is also a tetramer of molecular weight 110,000 (12). The reasons for the large size and subunit structure of the *B. megaterium* spore protease are not clear, but they could be related to its strict specificity for action on a macromolecular substrate (5, 15) (as is seen with collagenase). Indeed, studies on the interaction between the spore protease and its substrate proteins have indicated that the protease interacts with an extended region of the substrate protein's primary sequence (5). It is also possible that the subunit structure of the protease is important in the regulation of this enzyme, since the monomers appear to be inactive. Thus enzyme activity in vivo could be regulated by the degree of subunit association, which in turn could be modulated by various physiological factors (intracellular Ca^{2+} concentrations, pH, etc.).

Another rather unusual property of the spore protease is that its level appears to be regulated at least in part by proteolysis. The fall in spore protease activity during germination was previously ascribed to the ATP-dependent protein turnover which takes place during spore germination as well as in all other stages of growth (15, 17, 19). That protease antigen is lost in parallel with protease activity during germination is certainly consistent with a role for ATP-dependent proteolysis in this process.

The appearance of spore protease antigen only early in sporulation and only within the developing forespore indicates that this enzyme, like its substrates, is a spore specific gene product. Indeed, the time in sporulation at which the protease antigen appears is almost identical to, if not slightly before, the time of synthesis of the protease's substrates which also appear only in the forespore (14, 19). This finding raises an

obvious question concerning the regulation of the spore protease during this period, since the substrate proteins are not attacked until spore germination (14, 19). Thus the protease must be inactive in forespores and dormant spores, but active in germinated spores. Active spore protease has been extracted from forespores (15), but the enzyme could have been activated during extraction, and indeed any inactive enzyme extracted (i.e., zymogen) would have been undetected. Now with the availability of a sensitive assay able to detect inactive or monomeric forms (or both) of the protease, we should be able to examine in detail the mechanism for the regulation of this key proteolytic enzyme.

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