JOURNAL OF BACTERIOLOGY, June 1973, p. 1184-1192
Vol. 114, No. 3

Isolation of Two Acetyl Esterases from Extracts of *Bacillus subtilis*

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Received for publication 27 November 1972

Acrylamide gel electrophoresis of crude cellular extracts of *Bacillus subtilis* revealed the presence of two acetyl esterases. Esterase A, the slower migrating enzyme, was found to be present in both vegetative and sporulating cells, whereas esterase B activity was more abundant after exponential growth ceased. Both esterases were present in the supernatant fraction of lysed spheroplasts and in a disrupted spore preparation. Of four pleiotropic asporogenous mutants tested, three exhibited decreased esterase B activity. Esterases A and B were partially purified by differential precipitation and co-chromatographed on diethylaminoethyl (DEAE)-cellulose (pH 7.5) and DEAE-Sephadex (pH 8.5). By employing gel filtration chromatography, the two esterases were separated, and molecular weights of 160,000 and 51,000 were estimated for esterases A and B, respectively. Esterase A was further purified to electrophoretic homogeneity by differential heating and preparative starch block electrophoresis. Sodium dodecyl sulfate-acrylamide gel electrophoresis of purified esterase A yielded a single protein band with a molecular weight of 31,000. The pI values of esterases A and B were determined to be 6.4 and 5.4, respectively.

Recently, a variety of investigations has been concerned with the role of proteolytic enzymes in bacterial sporulation. In a number of these studies the characterization of extracellular proteases and esterases has been made. Three distinct extracellular enzymes have been isolated and identified in sporulating cultures of *Bacillus subtilis* (21, 25). One of the extracellular proteases is a neutral metalloenzyme which is active on protein but not on ester substrates (12, 16). The alkaline protease that belongs to the subtilisin class of endopeptidases possesses both proteolytic and esterolytic activity (7, 19, 26). The third enzyme, the acidic protease, does not attack protein substrates appreciably but has high esterase activity (4, 8, 21, 25). Since studies (17, 25) have suggested that these enzymes are also found intracellularly, several more direct attempts have been made to characterize the intracellular proteases (6, 22, 24).

Recently, Millet (22) reported the existence of a cytoplasmic endopeptidase synthesized by *B. megaterium* during sporulation. The enzyme was partially purified and found to possess high esterolytic and low proteolytic activity. Their results (23) indicated that this enzyme might be responsible for the interconversion of the deoxyribonucleic acid-dependent ribonucleic acid polymerase from that of the vegetative form to the sporulation form (15, 17). Previous studies by Leighton et al. (14) suggested that this interconversion could be accomplished by the action of the extracellular alkaline protease.

During the course of our investigation on the role of proteolytic enzymes in sporulation of *B. subtilis*, two intracellular esterases were identified. The aim of this study was to isolate the two esterases to determine their relationship to the proteolytic enzymes and bacterial sporulation.

MATERIALS AND METHODS

Organisms. Laboratory stock cultures of *B. subtilis* 168 (*trpC2*) were used for the production of esterase. Strain SR22 (*spoA12*, *trpC2*) was kindly supplied by J. Hoch as were high-exoprotease-producing strains, *hpr-12* and *hpr-97* (9; Higerd, Hoch, and Spizizen, in press). Strains L-4 and S-87 were a gift from J. Hageman. Strains TP33 (group B), TP73 (group A), TLA189 (group C), *absA6*, and *absB24* were isolated in this laboratory by J. Ito (10, 11; Ito and Spizizen, unpublished results).

Preparation of extracts. Cellular material used for the detection of esterase produced by the wild-type strain during growth was prepared as follows. Modified Schaeffer medium (13) containing (per liter): glucose, 1 g; nutrient broth (Difco), 16 g; KCl, 2 g;

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$CaCl_2$, 222 mg; $FeCl_3 \cdot 6H_2O$, 2.7 mg; $MnSO_4 \cdot H_2O$, 3.38 mg; and $MgSO_4 \cdot 7H_2O$, 24.65 mg, was adjusted to pH 7.0 with 10 N KOH and inoculated with 5×10^7 heavy spores of *B. subtilis* 168 (*trpC2*) per ml prepared by the method of Tamir and Gilvarg (33). Growth was followed turbidimetrically in a Klett-Summerson colorimeter equipped with the no. 66 red filter. Portions (50 ml) of the culture were then incubated at 37 C in 250-ml flanged-bottom flasks agitated at 250 rpm. At various time intervals, 50-ml samples were removed and centrifuged at 39,000 rpm for 15 min at 4 C after the percentage of refractile spores was determined by dark-phase photomicrographs of the cultures. The pellet was suspended in 1 ml of 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.5, containing 0.04 M $MgSO_4 \cdot 7H_2O$ and 0.3 mg of lysozyme per ml, frozen immediately in an ethanol-dry ice bath, and stored at −20 C overnight.

The crude esterase extract used as the starting material in purification was prepared from a 100-liter culture grown in a 150-liter-capacity fermentor (Fermentation Design, Inc., Allentown, Pa.). A trace amount of Dow Antifoam B was added to the modified Schaeffer medium prior to the addition of 2 liters of a 5-h culture of *B. subtilis* 168 (*trpC2*). Aeration was maintained at 4 cubic feet per min, agitation at 40, and temperature at 37 C. When growth reached 150 Klett units, the culture was passed through a Sharples refrigerated centrifuge, model AS16P, at 15,000 rpm. The cells were collected and washed three times in cold 0.1 M Tris-hydrochloride buffer, pH 7.5, containing 0.04 M $MgSO_4 \cdot 7H_2O$. The cells were resuspended in the same buffer (0.2 g [wet wt]/ml), lysozyme was added to give a final concentration of 0.3 mg/ml, and the mixture was stored frozen at −20 C.

For the detection of esterase, *B. subtilis* strain 168 (*trpC2*) and the mutants were grown on tryptose blood agar base (Difco) for 18 h at 37 C. The inoculum consisted of the suspended growth in 50 ml of modified Schaeffer medium, with an initial Klett reading of 30. After 8 h of incubation at 37 C, the culture was centrifuged and stored as described.

The frozen cell samples were thawed at room temperature and agitated at 37 C for 20 min. The lysozyme-treated cell suspension was placed in an ice bath and subjected to sonic disruption (Bronson, model S125) of three 30-s pulses at 1-min intervals. For maximal yield of esterase activity, 10 1-min pulses were used in preparing extracts for purification of the enzyme. The broken cell suspension was freed from cellular debris by centrifugation at 48,000 × *g* for 30 min at 4 C.

Esterase and protein assays. Esterase activity was assayed with β-naphthol acetate (recrystallized from dilute ethanol; Fischer Scientific Co., Pittsburgh, Pa.) as substrate, by a modification of the method of Seligman and Nachlas (30). To 0.5 ml of a test solution appropriately diluted in 0.1 M Trishyrochloride (pH 7.5), 2.5 ml of the substrate (0.1 mg/ml) in the same buffer was added, and the reaction mixture was incubated at 30 C. After 20 min, 0.5 ml of a freshly prepared solution of Fast Blue BB Salt (Sigma Chemical Co., St. Louis, Mo.; 4 mg/ml) was added, followed exactly 2 min later by the addition of 0.5 ml of 40% trichloroacetic acid. The resulting pigment was extracted by shaking the reaction mixture with 5 ml of ethyl acetate. To 2.5 ml of the organic layer, after centrifugation at 3,000 × *g*, an equal volume of ethyl acetate was added, and the color density was measured in the Klett-Summerson colorimeter equipped with a no. 54 green filter. From a calibration curve of pure β-naphthol, color density was converted to milligrams of β-naphthol. The unit of esterase activity was defined as milligrams of substrate hydrolyzed per 20 min at 30 C.

Determination of protein in esterase samples was based on the Zac and Cohen (36) modification of the Lowry method. Bovine serum albumin (Armour Pharmaceutical Co., Kankakee, Ill.) was used to prepare a standard curve.

Electrophoresis. A disk electrophoresis apparatus similar to that described by Davis (5) was used. The glass tubes were 5 mm internal diameter and 60 mm long. The height of the acrylamide gel column was 54 mm. The 7.5% acrylamide gel system was prepared as follows. Stock solution A contained acrylamide, 12 g; *N*,*N*′-methylenebisacrylamide, 320 mg; *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, 0.92 ml; and degassed 0.1 M Tris-hydrochloride (pH 7.5), to a volume of 80 ml. Stock B (prepared fresh daily) contained 140 mg of ammonium persulfate in 100 ml of the same buffer. The working solution was prepared by mixing equal parts of stock A and stock B. The acrylamide solution (1 ml) was placed in a glass tube, and buffer was carefully layered over the acrylamide solution. Polymerization of the acrylamide was completed after 60 min at room temperature. Both upper and lower reservoirs of the electrophoresis apparatus (5) were filled with the same buffer. The lower electrode served as the anode.

After polymerization of the acrylamide, the tubes were placed at 4 C, and 25 μliters of the test solution (containing 34.4 μg of protein in 20% sucrose) and a minute amount of bromophenol blue were carefully layered above the gel. Electrophoresis was carried out at 4 C until the tracking dye reached the end of the tube. After a loading amperage of 2 mA/tube for 15 min, a constant current of 10 mA/tube was maintained.

After electrophoresis, the gel was carefully removed from the electrophoresis tube where it was stained either for esterase activity or protein. The esterase staining solution consisted of 1 ml of β-naphthol acetate (20 mg/ml) in acetone, 150 mg of Fast Blue BB, and 99 ml of 0.1 M Tris-hydrochloride, pH 7.5. After 20 min of agitation, the gels were rinsed and stored in 7% acetic acid. The protein staining solution consisted of 0.2% Coomassie Brilliant Blue (Consolidated Laboratories, Inc., Chicago, Ill.), 50% methanol, 1% trichloroacetic acid, and 7% acetic acid. The gels were removed from the protein stain after 2 h, and the background was destained for 48 h against several changes of 30% methanol in 7% acetic acid. The gels were stored in 7% acetic acid.

Subunit determination was performed by heating the purified sample of esterase A at 80 C for 10 min in the presence of 1% sodium dodecyl sulfate (SDS) and

1% β-mercaptoethanol. The sample was cooled and applied in 20% sucrose to the surface of acrylamide gels to which SDS and mercaptoethanol had been incorporated prior to polymerization to a final concentration of 0.1%. The buffer in both reservoirs contained the same concentration of SDS and mercaptoethanol as the gels.

Enzyme purification. Partial purification and separation of the two esterases from cell-free extracts of *B. subtilis* were accomplished by the following procedures and unless noted otherwise were carried out at 4 C.

Protamine sulfate (2%) was added dropwise to the extract to give a final concentration of 0.4%. After 30 min, the mixture was centrifuged at 6,000 × *g* for 60 min, and the supernatant fluid was adjusted to pH 7.5 with 1 N NaOH. Solid ammonium sulfate was added to 55% saturation, and the mixture was refrigerated for at least 2 h. The precipitate was then centrifuged at 48,000 × *g* for 15 min and discarded. The supernatant fluid was brought to 85% ammonium sulfate, and the resulting precipitate was recentrifuged and suspended in 61.6 ml of 0.05 M Tris-hydrochloride, pH 7.5. After dialysis against 4 liters of the same buffer overnight, the solution was clarified by centrifugation at 48,000 × *g* for 15 min and chromatographed on a diethylaminoethyl (DEAE)-cellulose (Bio-Rad Laboratories, Richmond, Calif.) column which had previously been washed with 4 liters of 0.05 M Tris-hydrochloride buffer (pH 7.5). After the esterase sample was applied to the column (30 by 115 mm) for 1 h, 600 ml of the Tris-hydrochloride buffer was passed to eliminate any substances not readily adhering to the column material. Subsequently, 1 liter of a sodium chloride gradient (0 to 0.4 M sodium chloride in the 0.05 M Tris-hydrochloride buffer) was applied in a linear manner. The resulting chromatographic fractions containing esterase activity were concentrated by 85% ammonium sulfate precipitation, and the precipitate was suspended in 30 ml of 0.05 M Tris-hydrochloride (pH 8.5) and dialyzed against 2 liters of the same buffer. The solution was then applied to a DEAE-sephadex A-50 column (24 by 165 mm) equilibrated at pH 8.5 with 0.05 M Tris-hydrochloride buffer, and 300 ml of the Tris-hydrochloride buffer (pH 8.5) was passed through the column. The sodium chloride gradient was applied in the same manner. The resulting fractions exhibiting esterase activity were concentrated by 85% ammonium sulfate precipitation, and the precipitate was suspended in 0.1 M Tris-hydrochloride, pH 7.5.

Separation of the two esterases was achieved by Sephadex G-150 (Pharmacia Chemicals Inc., Uppsala, Sweden) gel filtration equilibrated with 0.1 M Tris-hydrochloride, pH 7.5. Samples of 1 ml were applied to a reverse-flow column (28 by 360 mm) and eluted with the same buffer, and 1.85-ml fractions were collected.

Esterase A was further purified as follows. The protein of the fractions containing the first peak of esterase activity was precipitated with ammonium sulfate (85%) and suspended in 1 ml of 1 M potassium phosphate buffer, pH 7.5. After overnight dialysis against 1 liter of the same buffer, the preparation was heated at 80 C for 10 min and cooled, and the resulting precipitate was centrifuged at 48,000 × *g* for 15 min. The supernatant fraction was applied to a small strip of Whatman no. 3 MM paper and partially dried. The sample paper was placed in horizontal trays containing 13% polymerized starch in borate buffer, pH 8.9, according to the formula of Smithies (32). Electrophoresis was carried out at a constant 200 V for 3 h. The starch strip was then sliced lengthwise and stained for esterase activity, as previously described, until the band first became visible. The strip was removed from the staining mixture and rinsed rapidly in distilled water. The activity band was carefully sliced from the gel, and the enzyme was eluted in 0.3 ml of 0.1 M Tris-hydrochloride, pH 7.5. This solution, resulting from preparative starch block electrophoresis, appeared to be electrophoretically pure and was designated purified esterase A.

RESULTS

Bott (3) had previously shown that cellular extracts of *B. subtilis* contained two esterases in varying amounts during sporulation. Our initial studies were designed to confirm and extend these results by using crude cellular extracts of *B. subtilis* undergoing sporogenesis. Modified Schaeffer medium was seeded with renografin-purified heavy spores (33) of *B. subtilis* 168 (*trpC2*), and the growth of the culture was monitored by measuring optical density. After 3 h of growth, samples of the culture were removed hourly and observed for refractile bodies. Additional samples were centrifuged, resuspended in 1/50 volume, and disrupted by treatment with lysozyme as well as sonic treatment. Each extract was diluted to an equivalent protein content and subjected to acrylamide gel electrophoresis.

Two esterases were distinguishable during the growth and sporulation cycle of the wild-type strain (Fig. 1B). One of the esterases appeared to be present throughout the growth cycle and had a slower migration rate. This esterase was arbitrarily termed "esterase A." The second esterase, designed "esterase B," migrated faster than esterase A and appeared to increase in amount later in the growth cycle and during sporulation (Fig. 1A).

To determine the presence of acetyl esterases in spores, disrupted spores of the wild-type strain were prepared by the method of Vold and Minatogawa (35). As is evident in Fig. 1B, spores share the same esterase patterns on acrylamide gels as the postexponential cells.

Electrophoresis of the extracts was also run under reverse polarity. However, in no case were discernible bands present when the gels were stained for esterase activity.

Mutants of *B. subtilis* blocked in the early

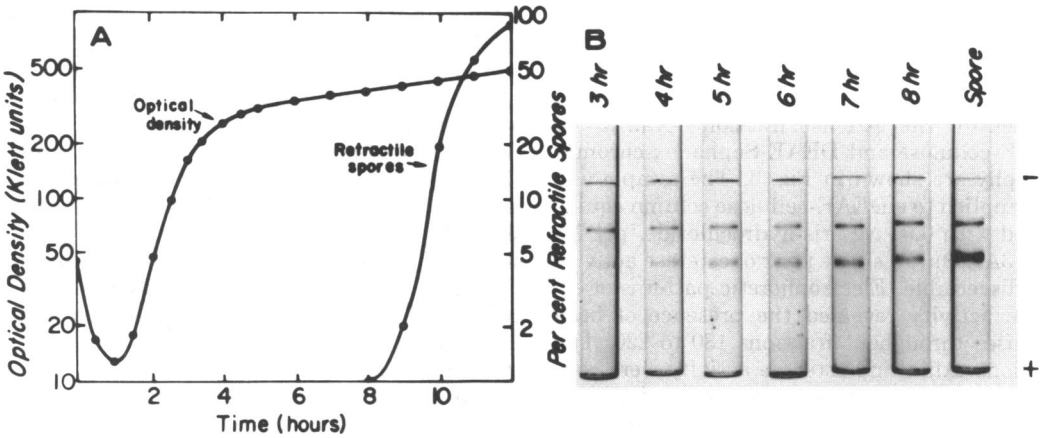

FIG. 1. *Kinetics of growth and appearance of refractile spores (A) in relation to histochemical staining of samples after acrylamide gel electrophoresis (B). Purified spores were inoculated into nutrient broth medium. At the indicated times, 50-ml samples were removed, optical density was read, and the percentage of refractile spores was observed. The pelleted cells were subjected to lysozyme treatment and sonic disruption. Electrophoresis and esterase staining were performed as described in Materials and Methods.*

stages of sporulation were shown previously by several workers (2, 9–11, 20) to produce little, if any, extracellular protease. Asporogenous mutants SR22 and TP73 which are mutated at the *spoA* locus (9, 11, 20) produced levels of esterase A comparable to wild type but lacked significant esterase B activity (Fig. 2). Mutant TP33, which maps at the *spoB* locus, showed a similar pattern of esterase activity. Strain TLA189, which is blocked at the same stage of sporulation but maps at the *spoC* locus, retained the esterase pattern of the parent strain.

Several additional mutants with altered protease levels were similarly tested (Fig. 2). Culture filtrates of strains *hpr-12* and *hpr-97* had previously been shown (9; Higerd and Spizizen, in press) to produce increased levels of extracellular proteases. The mutants, however, appeared to produce normal levels of the intracellular esterases. Strains L-4 and S-87 which are markedly deficient in an intracellular neutral protease and a "new" protease, respectively, (Hageman and Carlton, in preparation) produced esterase bands similar to the wild-type strain.

In an attempt to determine the cellular localization of the esterases, we prepared spheroplasts by suspending washed cells in an equivalent volume of 0.1 M Tris-hydrochloride buffer (pH 7.5) containing 0.5 M sucrose and 1 mg of lysozyme per ml. After 10 min at 37 C, the culture was observed by microscopy to contain greater than 95% spheroplasts. The spheroplasts were centrifuged at 6,000 × *g* for 10 min and suspended in an equal volume of cold 0.1 M Tris-hydrochloride, pH 7.5. The resulting lysate

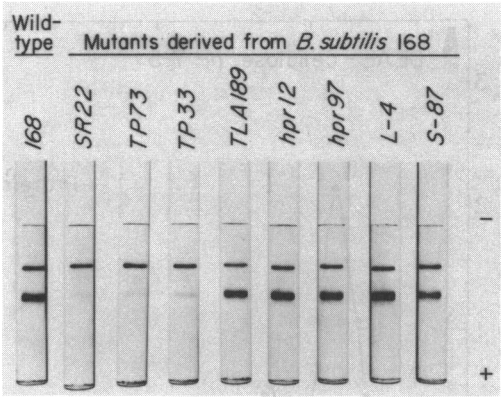

FIG. 2. *Histochemical staining for acetyl esterase activity following acrylamide gel electrophoresis of crude extracts from wild type and mutants of B. subtilis. The strains were grown in nutrient broth medium for 8 h prior to disruption of the harvested cells. The soluble fraction was subjected to electrophoresis.*

contained cellular debris and was recentrifuged at 40,000 × *g* for 20 min.

The precipitate and other samples removed during the procedure, i.e., whole cell washings, spheroplast washing, etc., contained approximately 6% of the total activity recovered. The remaining 94% of the activity was found in the supernatant fluid after lysis and centrifugation of the spheroplasts. Since the supernatant fraction yielded a similar electrophoretic pattern of esterase activity as did sonically treated whole cells, it can be assumed that both esterase A and B are localized in the cytoplasm.

Partial purification of the two acetyl esterases was accomplished by DEAE-chromatography of the protamine sulfate and ammonium sulfate-fractionated extracts as described. The elution profiles for the esterase fraction obtained by DEAE-cellulose and DEAE-Sephadex chromatography are shown in Fig. 3. The sample was first applied to a DEAE-cellulose column equilibrated with 0.05 M Tris-hydrochloride, pH 7.5 (Fig. 3A). Only a single peak of esterase activity was discernible. Electrophoretic patterns of esterase activity revealed the presence of both esterases throughout fractions 180 to 220. The fractions exhibiting esterase activity were concentrated by ammonium sulfate precipitation and applied to a DEAE-sephadex column equilibrated with 0.5 M Tris-hydrochloride, pH 8.5 (Fig. 3B). Esterase activity was eluted as one peak which exhibited an electrophoretic pattern of esterase activity similar to the crude extract.

The two enzymes were purified approximately 16-fold with a recovery of 39% of the total activity (Table 1) prior to Sephadex G-150 chromatography which partially separated the two esterases. Two peaks of esterase activity were discernible and did not correspond to the two main protein peaks (Fig. 4). Several fractions containing esterase activity were subjected to acrylamide gel electrophoresis and stained for esterase activity (Fig. 4B). The slower-migrating esterase corresponds to the first eluted activity peak of the Sephadex G-150 column, whereas the leading esterase band on electrophoresis corresponds to the lower-molecular-weight esterase.

Proteins of known molecular weight were applied to the same column to determine the relative molecular weights of the esterases. Figure 5 depicts the results of the standardization, and molecular weights of 160,000 and 51,000 were estimated for esterases A and B, respectively. (Molecular weight estimation from

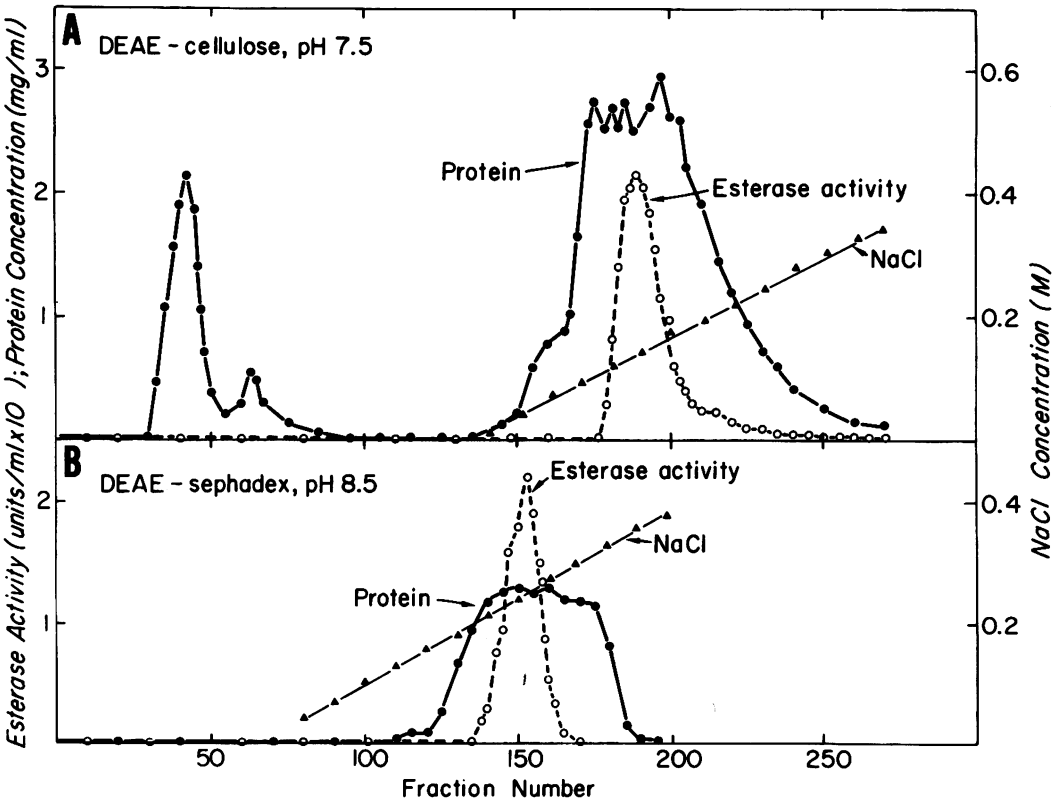

FIG. 3. *Elution profile of esterase activity and protein on DEAE-cellulose and DEAE-Sephadex equilibrated in 0.05 M Tris-hydrochloride buffer at the indicated pH. After the passage of starting buffer, a linear NaCl gradient was established. Each fraction contained 6.1 ml, and the flow rate was maintained at 44 ml/h. NaCl concentration was monitored by measuring the electrical conductivity of the solution relative to a standard curve titrated with known concentrations of NaCl in the same Tris-hydrochloride buffer. Esterase activity and protein concentration were assessed as described in Materials and Methods.*

TABLE 1. *Purification of total esterase activity prior to Sephadex G-150 chromatography*

Stage	Total vol (ml)	Total enzyme (U × 10^6)	Total protein (g)	Specific activity[a]	Yield (%)
Crude extract	835	2.87	5.64	510	100
Protamine sulfate	962	2.45	2.51	975	85
55% ammonium sulfate	1,132	2.25			78
85% ammonium sulfate	61.6	1.90	1.36	1,405	66
Dialysis I	106.2	1.67	1.15	1,451	58
DEAE-cellulose eluate	212	1.57	0.46	3,437	55
Dialyzed ammonium sulfate precipitate	35.1	1.59	0.44	3,621	56
DEAE-Sephadex eluate	134	1.12	0.13	8,522	39

[a] Specific activity, units of esterase activity per milligram of protein.

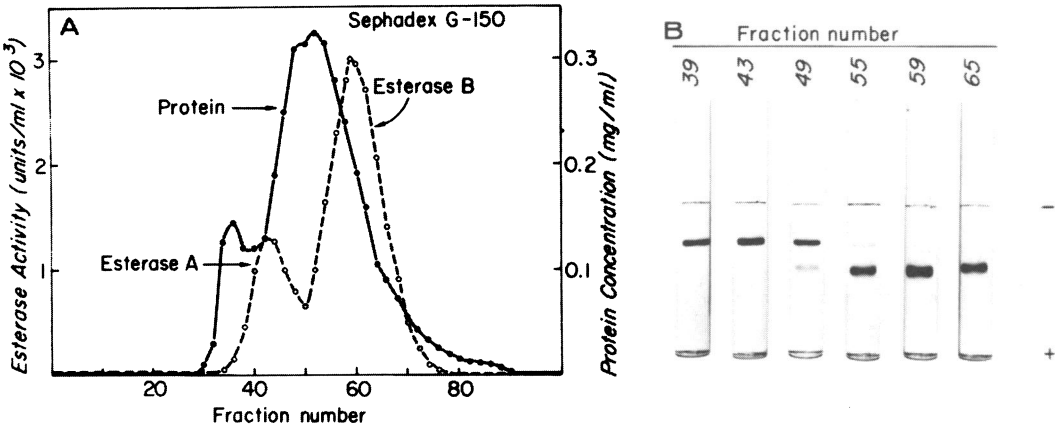

FIG. 4. *Partial separation of esterases A and B by Sephadex G-150 chromatography (A). The characteristics of the column are described in Fig. 5. Several fractions were subjected to acrylamide gel electrophoresis and stained for acetyl esterase activity (B).*

a standardized Sephadex G-100 column yielded 130,000 and 46,000 for esterases A and B, respectively.)

Fractions 36 to 40 (Fig. 4A), containing esterase A activity, were combined and concentrated by ammonium sulfate precipitation. Likewise, fractions 60 to 67, containing esterase B activity, were combined and concentrated. The precipitates were suspended in a small volume of 0.1 M Tris-hydrochloride buffer, pH 7.5, and heated for 10 min at the temperatures indicated in Fig. 6A. At 60 C, esterase A retained all of its original activity upon cooling while esterase B was found to be totally inactivated at the same temperature. After suspending the ammonium sulfate precipitate of the esterase A fractions in various concentrations of potassium phosphate buffer, pH 7.5, and heating the preparation at 80 C for various times (Fig. 6B), the following was observed. Esterase A activity appeared to be protected with increasing concentrations of phosphate buffer. On the other hand, esterase B (not shown in Fig. 6B) was as labile in phosphate buffer as in the Tris-hydrochloride buffer.

By heating the esterase A solution and removing the denatured proteins by centrifugation, a solution was obtained that had a specific activity of approximately 3 × 10^5 U/mg of protein. Additional contaminating proteins were removed by preparative starch electrophoresis as described. Acrylamide gel electrophoresis of samples from the last purification steps are presented in Fig. 7. The solution which produced a single protein band with a corresponding activity band was considered a pure esterase A preparation.

To determine the subunit composition of esterase A, the purified solution of the high-molecular-weight enzyme was heated in the presence of SDS and mercaptoethanol and subjected to electrophoresis in acrylamide gels

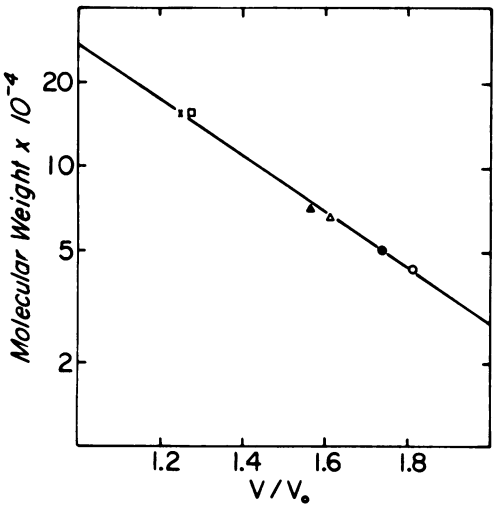

FIG. 5. *The relationship between elution volume (V) from a Sephadex G-150 column and the molecular weights of several proteins. The buffer employed was 0.1 M Tris-hydrochloride, pH 7.5. The column void volume (V_o = 34.2 ml) was determined from the elution volume of Blue Dextran 2000 (five determinations; range, ±0.4 ml). All elution volumes were established by protein assay of effluent fractions. Symbols: ×, esterase A; □, bovine gamma globulin; ▲, transferrin; △, bovine serum albumin; ●, esterase B; ○, ovalbumin.*

containing SDS and mercaptoethanol. The migration distance was recorded relative to the migration distance of proteins of known molecular weights (Fig. 8). Only a single protein band was observed with an estimated molecular weight of 31,000.

The isoelectric point of the two esterases was determined by the method of Vesterberg (34). Purified esterase A and partially purified esterase B solutions were applied to acrylamide gels containing ampholite, pH 3 to 10. After electrophoresis, the gels were removed and stained for esterase activity. Esterase A activity was present as a broad band, whereas esterase B activity was confined to a narrow band. The pH of 1-mm sections of a duplicate gel was determined and pI values of esterases A and B were estimated to be 6.4 and 5.4, respectively.

DISCUSSION

The presence of esterases using nonspecific ester substrates has been reported previously in crude extracts of vegetative cells (1, 3) and spores (27, 28, 31) of *Bacillus* species. Roberts and Rosenkrantz (27, 28) were able to measure acetyl esterase activity in both intact and ruptured preparations of *B. cereus* and *B. coagulans* spores. Recently, Millet (22) reported the existence of a cytoplasmic endopeptidase of *B. megaterium* that possessed esterase activity towards the ethyl ester of benzoyl tyrosine and the *p*-nitrophenyl ester of carbobenzoxytyrosine. The relationship of these esterases to esterases A and B is not known.

During the course of this investigation, Bott (3) reported the existence of two esterases during the early stages of sporulation in *B. subtilis* as revealed by acrylamide gel electrophoresis of intracellular extracts. Our results (Fig. 1) appear to be comparable to his. In both cases, the leading esterase band (esterase B) appeared prior to the time of spore commitment (time zero; reference 29). Since this activity was low in the early blocked sporulation mutants, SR22, TP73, and TP33, its occurrence in wild-type cell extracts prior to the estimated commitment time may be an indication of asynchronous commitment of the population.

It is of interest to note that the asporogenous mutants, SR22, TP73, and TP33, which produced diminished levels of extracellular proteases (9; Ito and Spizizen, in preparation), produced less intracellular esterase B activity but normal levels of esterase A activity. This would suggest that esterase A activity, which appears to be present during the vegetative phase of growth, may not be involved in sporulation. The function of esterase B in the cellular differentiation cycle of *B. subtilis* awaits further investigation.

Mutants of the early, blocked asporogenous strain SR22 that are resistant to antibiotic(s) produced by *B. subtilis* 168 have previously been isolated in this laboratory (10, 11). These mutants (*abs*) remain asporogenous, but two of the four phenotypic groups of these mutants regained extracellular protease production. This mutation was shown (11) to be a secondary mutation. Strains *absA6* and *absB24* were tested for the presence of intracellular esterases and were found (unpublished results) to contain approximately normal levels of esterases A and B.

Esterases A and B were tested (unpublished results) for protease activity by using azocasein as the substrate. Neither a purified preparation of esterase A containing 27,000 U/ml nor a partially purified preparation of esterase B containing 1,000 U/ml appreciably hydrolyzed azocasein under the conditions used (21). The enzyme reported by Millet (22) and Millet et al. (23) possessed high esterolytic and low proteolytic activity. Likewise, the extracellular serine protease (acid protease) which is produced during sporulation of *B. subtilis* is highly active on ester substrates but has low activity as measured with protein substrates (4, 8, 21, 25).

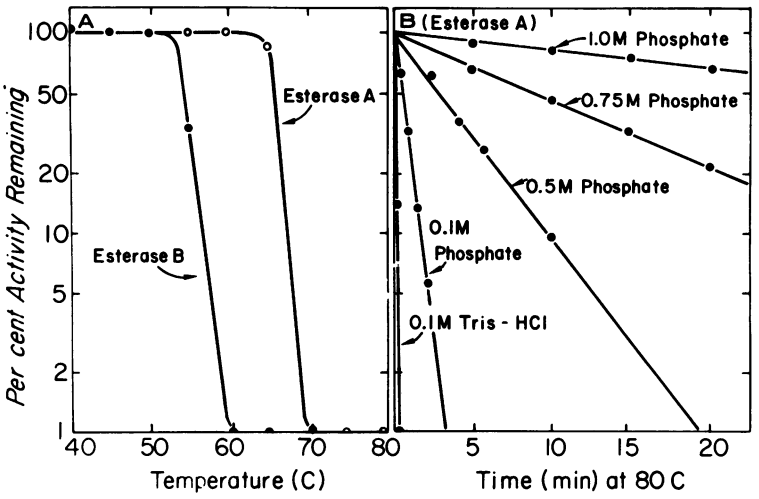

FIG. 6. *Heat inactivation of acetyl esterases at various temperatures for 10 min (A) and heat inactivation of esterase A incubated for various times at 80 C in either 0.1 M Tris-hydrochloride (pH 7.5) or phosphate buffer (pH 7.5) at different concentrations (B). The heated solutions were immediately cooled in an ice bath and assayed at 30 C without removal of the precipitate. Prior to heating, samples of the preparation were dialyzed against the appropriate buffer for 18 h at 4 C and assayed in the same buffer as the dialysate.*

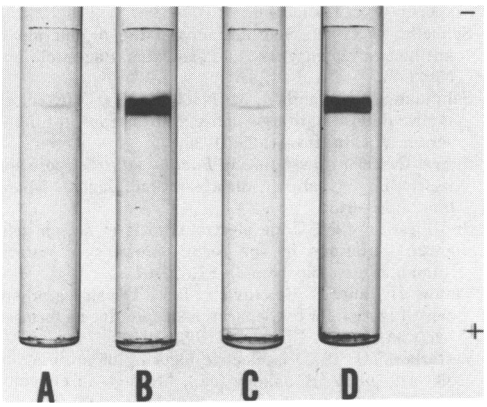

FIG. 7. *Acrylamide gel patterns after electrophoresis of clarified heat-treated preparation (gels A and B) and the purified solution of esterase A (gels C and D) obtained by subsequent preparative starch electrophoresis. Gels A and C were stained for protein (Coomassie Brilliant Blue), whereas gels B and D were stained for esterase activity.*

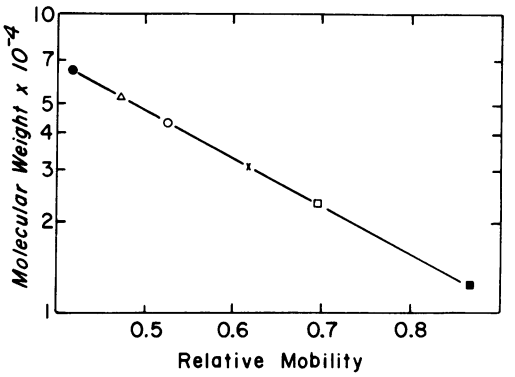

FIG. 8. *Determination of the molecular weight of purified esterase A in the presence of 0.1% SDS. The preparation of enzyme and markers were first heated at 80 C for 10 min in SDS and mercaptoethanol, followed by electrophoresis in acrylamide gels containing SDS and mercaptoethanol (see Materials and Methods). The mobility was measured relative to bromophenol blue. Symbols: ●, bovine serum albumin; △, B. subtilis phage φ29 main head protein (gift to J. Ito); ○, ovalbumin; ×, esterase A; □, trypsin; ■, cytochrome C.*

Further studies are needed to prove the identity of these enzymes with esterases A or B, or both, but the similarities in their enzyme activities and isolation procedures suggest that indeed they may be identical.

ACKNOWLEDGMENTS

We thank J. Hageman and J. Hoch for allowing us to test their strains and acknowledge our debt to J. Ito, I. P. Crawford, and V. Gage for their help and advice.

T. B. H. was a recipient of a Dernham Junior Fellowship of the American Cancer Society, California Division (no. 189).

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