JOURNAL OF BACTERIOLOGY, July 1983, p. 145–152
0021-9193/83/070145-08$02.00/0

Vol. 155, No. 1

Bacillopeptidase F: Two Forms of a Glycoprotein Serine Protease from *Bacillus subtilis* 168

CAROLYN A. ROITSCH† AND JAMES H. HAGEMAN*

Department of Chemistry, New Mexico State University, Las Cruces, New Mexico 88003

Received 17 February 1983/Accepted 23 April 1983

Bacillopeptidase F is a serine endopeptidase excreted by *Bacillus subtilis* 168 after the end of exponential growth. As a step toward discovering a physiological function for this protease, an enzymological and immunological study was undertaken. When bacillopeptidase F was purified at pH 10, a number of enzymically active, rapidly moving electrophoretic forms were observed, as had been previously reported. Rabbit antiserum was prepared against one form. When the enzyme was purified at pH 6.0 in the presence of the covalent inhibitor phenylmethylsulfonyl fluoride, using the rabbit antiserum to detect the bacillopeptidase F protein, no fast-moving electrophoretic forms were observed. Instead, only two forms of the enzyme were isolated. One form had a molecular weight of 33,000, and the other had a molecular weight of 50,000, as determined by equilibrium sedimentation methods. Both forms appeared to be glycoproteins, both contained compounds, released on acid hydrolysis, which cochromatographed with phosphoserine and galactosamine, and the two gave identical immunoprecipitin lines in Ouchterlony double-diffusion tests. The smaller form had a pI of 4.4, whereas the larger had a pI of 5.4. The data suggest that bacillopeptidase F is distinct from all other proteases of *B. subtilis*.

Intracellular proteolysis is one of the major metabolic events which occurs during bacterial sporulation (16), and one or more endopeptidases is presumed to be involved in this process. We undertook an immunological, enzymatic, and physical characterization of one protease activity potentially involved in the proteolysis associated with the sporulation of *Bacillus subtilis* 168.

After the end of exponential growth, *B. subtilis* 168, a genetically transformable strain, and other closely related strains excrete three proteolytic enzymes. The first is an endopeptidase of the serine type referred to variously as alkaline protease (18, 19, 35), enzyme A (24), basic protease, or bacillopeptidase E (2, 10; see table in reference 10 for a comparison of this protease with subtilisins). The second activity is a zinc-requiring endopeptidase referred to as the neutral protease (18, 34, 36) or enzyme B (24). The third enzyme activity, the subject of this report, is also a serine-type endopeptidase referred to as acidic protease (2), esterase (16, 19), enzyme C (24), enzyme II (15), or bacillopeptidase F (10).

Bacillopeptidase F is the least well characterized of the three proteases. Some substrate specificity and kinetic properties have been described (10), and a molecular weight of 36,000 (sedimentation rate) has been reported for a highly purified preparation (2). Several authors have reported more than one band of activity upon electrophoresis or chromatography of bacillopeptidase F (11, 15, 24), but no cause for this multiplicity of forms has been suggested. Some evidence has suggested that this protease may be found intracellularly (11, 24) or mesosomally (6) as well as in culture broths of *B. subtilis*, but no physiological function has been established for the enzyme.

In the presence of a covalent inhibitor, we have been able to isolate two immunologically similar forms of the enzyme. We present evidence that the multiple forms previously observed may have arisen from autodigestion. Both forms of the enzyme are shown to be glycoproteins, and their isoelectric points, molecular weights, and amino acid compositions are reported. Some of these results have been presented elsewhere (C. A. Roitsch and J. H. Hageman, Fed. Proc. 35:1474, 1976).

MATERIALS AND METHODS

Chemicals. Media used for bacterial maintenance and growth, TAM sporulation agar, Penassay broth dehydrated, gelatin, neopeptone, yeast extract, Bacto-Agar, special agar-Noble for immunoelectrophoresis, and Freund complete and incomplete adjuvant were obtained from Difco Laboratories.

† Present address: Basel Institut für Immunologie, Postfach CH-4058, Basel 5, Switzerland.

N-Benzoyl-L-tyrosine ethyl ester was purchased from Calbiochem, casein (Hammersten quality) was purchased from Nutritional Biochemicals Corp., and azocasein sulfanilamide was purchased from Sigma Chemical Co.

Phenylmethylsulfonyl fluoride (PMSF), riboflavin, 2-mercaptoethanol, *N,N,N',N'*-tetramethylethylenediamine, EDTA, basic fuschin stain, all the zwitterionic buffers of Good et al. (9), CHES [2-(*N*-cyclohexylamino)ethane sulfonic acid], MES (morpholineethanesulfonic acid), MOPS (morpholinepropanesulfonic acid), and Tris were obtained from Sigma. Enzyme-grade ammonium sulfate was purchased from ICN Pharmaceuticals Inc. Cellulose powder CC 31 and DEAE-cellulose preswollen microgranular anion exchanger were purchased from Whatman. Acrylamide and bisacrylamide, gold label, electrophoresis-grade 99+%, were purchased from Aldrich Chemical Co. The sodium dodecyl sulfate (SDS) was reagent grade from Sargent Welch Scientific Co. and was crystallized three times from 95% ethanol before use. Ultrapure urea was purchased from Schwarz/Mann. Isoelectric focusing ampholines, pH ranges 3.5 to 10, 5 to 7, and 4 to 6, were from LKB Instruments Inc. The Coomassie brilliant blue G-250 was from Bio-Rad Laboratories. The Dowex resin used in diffusion destaining gels was analytical-grade mixed-bed resin AG 501-X8, 20 to 50 mesh fully regenerated, from Bio-Rad.

Proteins used as standards for SDS-urea gel electrophoresis were: bovin serum albumin, fraction V, 96 to 99% albumin; catalase from bovine liver, purified powder, thymol free; ovalbumin grade V, electrophoretic purity, approximately 99%; pepsin from hog mucosa, twice crystallized and lyophilized; carbonic anhydrase from bovin erythrocytes; cytochrome *c* from horse heart, type VI, assay 97%; and insulin from bovine pancreas, crystalline (all from Sigma). Chromatographic-grade RNase was obtained from P-L Biochemicals.

Fluorocarbon, FC 43, for sedimentation equilibrium ultracentrifugation, was obtained from Beckman Instruments, Inc. Spectroscopic type IIG (2 by 10 by 0.04 in [ca. 5.08 by 25.4 by 0.102 cm]) glass plates for recording interference data on a Beckman model E ultracentrifuge were purchased from Eastman Kodak Co. D-19 developer and Rapid Fix from Kodak were used in the photographic plate development.

Bacterial maintenance, growth, and harvest procedures. The transformable tryptophan-requiring auxotroph *B. subtilis* 168 (Trp^-) (27) was used throughout these studies. Permanent stock cultures were stored on TAM sporulation agar slants at 4°C. Working stocks were maintained on gelatin-agar plates which contained 1.2% (wt/vol) gelatin, 0.4% (wt/vol) neopeptone, 0.1% (wt/vol) yeast extract, and 1.5% (wt/vol) Bacto-Agar.

For a given growth experiment, a single well-sporulated colony from a dehydrated gelatin-agar plate was used to inoculate 10 ml of Penassay growth as a preinoculum which was incubated for 8 h at 30°C and 200 rpm in a New Brunswick G-86 water bath shaker. A total of 6.5 ml of the Penassay culture was added to 150 ml of 2% (wt/vol) yeast extract in a 300-ml baffled shake flask and incubated for 2 h at 37°C and 200 rpm. For the final inoculation, 10 ml of this culture was added to 500 ml of 2% (wt/vol) yeast extract in 2-liter baffled shake flasks (Bellco Glass, Inc.), and the culture was incubated for 24 h at 37°C and 200 rpm in a New Brunswick G-25 shaker. Growth of the cultures was measured turbidimetrically by using a Klett-Summerson photoelectric colorimeter with a no. 66 filter. Cells were harvested in a CEPA Laboratory flow-through centrifuge and discarded. The culture broth was retained for protease purification.

Protease purification. Six liters of cell-free culture broth was chilled at 8°C for 12 h. The bacillopeptidase F activity was partially purified by precipitation onto cellulose powder with 65% (wt/vol) ammonium sulfate and eluted from DEAE-cellulose by procedures previously described (2).

The pooled bacillopeptidase F fractions (18 mg in 55 ml) were dialyzed against 0.235 M Tris-phosphate (pH 7.2) and applied to a preparative-scale polyacrylamide gel. A Buchler Poly-Prep electrophoresis apparatus was used by the modified method (5, 19) outlined in the manufacturer's operating instructions. The electrophoresis chamber was cooled with a Haake FK constant-temperature circulating water bath operated at 4°C, and fractions were collected at 8°C with a Sigmamotor peristaltic pump. Current was maintained between 30 to 50 mA during the run, which required a total of 20 h. Active fractions were dialyzed for 7 h with five buffer changes against sodium carbonate (pH 10) or phosphate buffer (pH 6) to obtain accurate Lowry protein determinations.

Enzyme assays. Proteolytic activity was measured by detecting release of acid-soluble products from azocasein as previously described (7).

Azocasein assays for proteolytic activity in sliced polyacrylamide gels contained 1.0 ml of 1% (wt/vol) azocasein in 0.1 M Tris-chloride (pH 7.8) plus the gel slice being assayed. Incubation times were extended to 12 h at 37°C to allow enzyme to diffuse from the gel. Spectrophotometric determinations of the hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester were carried out as described previously (2).

Ouchterlony immunoprecipitin assay. Bacillopeptidase F, inhibited with PMSF, was monitored semiquantitatively by an immunoprecipitin assay (21). Petri plates were filled with immunodiffusion agar (10) to a height of 5 to 10 mm. The central well was filled with antiserum against bacillopeptidase F, and fractions eluting from various columns were placed in one of six outer wells. Fractions containing bacillopeptidase F formed a precipitin line at the equivalence point of the antibody-antigen system after incubation for 12 h at room temperature. Consecutive fractions exhibiting identical precipitin bands or cross-reacting material were analyzed electrophoretically to determine their homogeneity. Electrophoretically homogeneous fractions were then pooled.

Protein determinations. Protein fractions in buffers other than Tris were assayed by using the Folin phenol reagent (14). Protein fractions which were in Tris buffers were assayed by the Coomassie brilliant blue G-250 dye-binding assay (Bio-Rad kit) or by measuring the ratio of the optical density at 280 nm to that at 260 nm and using the formula $1.45\ A_{280} - 0.74\ A_{260}$ = milligrams of protein per milliliter (where A is the absorbance at 280 and 260 nm) (13) to calculate protein concentrations.

Polyacrylamide gel electrophoresis. Standard analytical 7 and 10% acrylamide gels were prepared by the

methods of Davis (5) and Ornstein (20) with gel tubes (5 mm [inner diameter] by 90 mm) in a Shandon SAE-270 Mk II apparatus. Analytical gels were also prepared by the method used for preparative electrophoresis. In both cases, proteins were detected by staining gels in 0.25% (wt/vol) Coomassie brilliant blue G-250 in 7% acetic acid for 12 h. Gels were destained in 7% acetic acid in a diffusion destainer (model 172A, Bio-Rad) charged with AG 501-8X resin.

Preparation of bacillopeptidase F antiserum. A 3-kg male rabbit was given weekly intramuscular injections of 1.7 mg of bacillopeptidase F (peak A of Table 1) for 10 weeks. A solution of peak A (1 to 1.5 ml) was mixed with an equal volume of Freund complete adjuvant for the initial injection; for each subsequent injection, a solution of peak A (1 to 1.5 ml) was mixed with an equal volume of Freund incomplete adjuvant. Beginning 3 weeks after the initial injection, the rabbit was bled periodically by cardiac puncture. The crude antiserum was separated from the clotted blood and stored at −10°C in 5-ml portions without additives.

Determination of molecular weight by sedimentation equilibrium. The molecular weight of bacillopeptidase F was estimated by the meniscus depletion sedimentation equilibrium method (4, 39). A solution of approximately A_{280} = 0.5 was dialyzed for 4 h with four changes of buffer (50 mM sodium acetate, 0.1 M KCl [pH 6.0]). Runs were allowed to come to equilibrium by centrifugation for 18 to 24 h at speeds from 20,000 to 28,000 rpm. The interference data were read by using a microcomparator, a Mann measuring engine interfaced with a Baldwin Shaft Position Encoder (model 5V78D). Each plate was read at least twice. Molecular weights were determined by the method of Yphantis (39) by linear regression analysis. The logarithm of the y-displacement of the interference fringes, which gave correlation coefficients of 0.992 or better, was used.

Determination of molecular weight by electrophoresis in SDS-urea gels. Molecular weights were determined on polyacrylamide gels containing 0.1% (wt/vol) SDS, 0.1 M H_3PO_4, and 8 M urea, with a final pH of 6.8, by the method of Swank and Munkres (33). Two-milliliter gels of 8 or 12.5% acrylamide were run in Pyrex tubes (5 mm [inner diameter] by 130 mm) at 3 to 4 mA per gel for 3 to 5 h; PMSF (2 to 3 mM) was included in preincubation solutions. A 3% acrylamide stacking gel was prepared for some experiments. Standard proteins (listed above) were electrophoresed in each experiment to construct linear standard curves.

Assays for glycoproteins and lipoproteins. The presence of glycoproteins was tested by the procedure of Zacharius et al. (40) after electrophoresis in analytical polyacrylmide gels. Ovalbumin and human transferrin were used as glycoprotein standards; bovine serum albumin and carbonic anhydrase were used as nonglycoprotein standards. Control gels, not treated with periodic acid, did not react with basic fuchsin. Tests for lipoprotein were made by the method of Ressler et al. (25).

Determination of amino acid composition. Fractions from peak 1 or peak 2 which gave homogeneous bands on 10% polyacrylamide gels were analyzed. Fractions were dialyzed against deionized water containing 0.04% sodium azide to try to remove glycine (present in the electrophoresis buffer). Dialyzed samples were hydrolyzed in 5.7 M HCl at 110°C for 24 to 26 h in evacuated tubes. Analyses were made on a Beckman 121 M or Durrum 400 automatic amino acid analyzer. Even after extensive dialysis, apparently high levels of glycine were measured (see footnote *f* of Table 3).

TABLE 1. Purification of bacillopeptidase F at pH 10 in the absence of PMSF

Fraction	BTEE units[a]	BTEE units/mg	% Yield	Purification
Culture broth	53,900	3	100	1
$(NH_4)_2SO_4$-cellulose column elutant	10,600	16	20	5×
Dialyzed elutant	11,300	31	21	10×
DEAE-cellulose elutant	7,500	420	14	143×
Sample applied to preparative PAGE[b]	6,800	394	13	116×
Pooled fractions from preparative PAGE				
Peak A	2,900	540	5	168×
Peak B	2,400	478	4	149×

[a] One *N*-benzoyl-L-tyrosine ethyl ester (BTEE) unit equals an optical density of 0.001 at 256 nm after 1 min at 22°C in 0.1 M potassium phosphate (pH 7.6).

[b] PAGE, Polyacrylamide gel electrophoresis.

RESULTS

Production and isolation, at pH 10, of bacillopeptidase F in the absence of a covalent inhibitor. Cells of *B. subtilis* 168 began to excrete bacillopeptidase F after 10 to 12 h of growth in 2% yeast extract, as shown in Fig. 1. The final step of our abbreviated purification scheme (Table 1), a preparative-scale polyacrylamide electrophoresis, yielded two apparently homogeneous proteins (Fig. 2). Both were enzymatically active toward *N*-benzoyl-L-tyrosine ethyl ester and azocasein. The specific activities of these on the ester substrate were similar to that reported earlier for highly purified acidic protease (2). Peak A of Table 1 corresponds to the faster-moving protein band of Fig. 2, and peak B corresponds to the slower moving band.

Upon storage, the peak A fraction gave rise to several proteolytically active electrophoretic species. For example, electrophoresis of a peak A fraction from one preparative polyacrylamide gel column revealed one major protein band and one band of proteolytic activity, both having an R_f of 0.43. After 5 days of storage at 4°C and pH 10, electrophoresis of the same fraction on 10% polyacrylamide gels revealed four major protein bands (R_f: 0.42, 0.49, 0.61, and 0.81) and four major azocaseinase activities (R_f: 0.38, 0.52, 0.65, and 0.81). Mamas and Millet (15) have previously reported that their most highly purified fractions of this enzyme also contained four proteolytically active electrophoretic bands. In all the work carried out at pH 10, upon storage

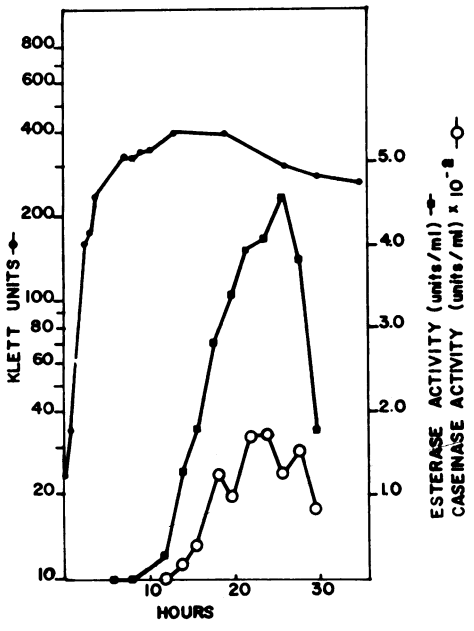

FIG. 1. Growth curve and appearance of esterolytic and proteolytic activity for *B. subtilis* 168 in 2% (wt/vol) yeast extract. At the times shown by the points, 20-ml samples were sterilely removed from the culture, centrifuged, and concentrated 10-fold with an Amicon PM 10 ultrafilter. Esterase and caseinase activity were assayed as described in the text, with 0.1 ml of the concentrated supernatant solution per assay.

and workup, active proteolytic bands with R_fs similar to those cited were repeatedly observed.

Determination of pH activity profile of bacillopeptidase F. The above results suggested that a pH of 10, adopted by Boyer and Carlton (2), was perhaps promoting autodigestion. This had not been expected, since (i) no loss in total activity was observed at pH 10, and (ii) pH 10 was well beyond the pH optimum of 7.5 to 8.0 reported for the hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester (2, 15). However, bacillopeptidase F was found to be extremely active at pH 10 when azocasein was used for the assay in a ternary buffer (17 mM each of MES, MOPS, and CHES). A threefold increase in azocaseinase activity (see Materials and Methods) for peak A enzyme was observed between pH 6 and 9.8. Thus, under this basic isolation condition, autodigestion was possible.

Preliminary attempts were made to purify the enzyme at pH 6.0. Even at this pH, however, multiple, fast-moving electrophoretic forms were detected. Thus, it was decided to attempt to purify covalently inhibited enzyme by using antiserum against bacillopeptidase F to detect the enzyme protein.

Preparation of bacillopeptidase F antiserum. A fraction from peak A (Table 1) was injected into rabbits, and antiserum was collected (Materials and Methods). Control antiserum did not cross-react against bacillopeptidase F, but antisera from inoculated rabbits gave strong cross-reactions against bacillopeptidase F on standard Ouchterlony immunodiffusion plates (21). Bacillopeptidase F antisera did not cross-react with bacillopeptidase E, the subtilisin-like enzyme from *B. subtilis* 168 (data not shown). This result was expected, as it had been shown earlier that bacillopeptidase E antiserum did not cross-react with bacillopeptidase F (11).

It is noteworthy that at least three of the fast-moving electrophoretic forms which arose from peak A as described above gave indistinguishable immunoprecipitin lines (Fig. 3). This precipitin pattern was confirmed by eluting these bands from a 10% preparative gel and observing that all three proteins gave precipitin lines of identity in an Ouchterlony double-diffusion plate (data not shown).

Purification of bacillopeptidase F at pH 6.0 in the presence of PMSF. To minimize possible autodigestion, we isolated bacillopeptidase F as described above but in 0.05 M potassium phosphate buffer at pH 6 in the presence of PMSF. We first demonstrated that a preparation of uninhibited bacillopeptidase F did not lose enzymatic activity at pH 6. It was also necessary to add PMSF after each chromatographic step, especially electrophoresis, as the latter promoted the partial hydrolysis of the phenylsulfonate ester of the protease and restored some catalytic activity. To follow the purification of bacillopep-

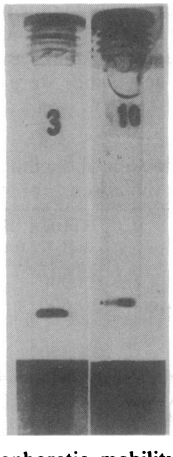

FIG. 2. Electrophoretic mobility of two forms of bacillopeptidase F. A 40-μg amount of peak A (gel 3) and 13 μg of Peak B (gel 10) from the purification shown in Table 1 were electrophoresed on 7.0% polyacrylamide gels (5, 20) and stained for protein as described in the text. The R_f values with respect to the dye fronts were 0.97 and 0.88 for peaks A and B, respectively.

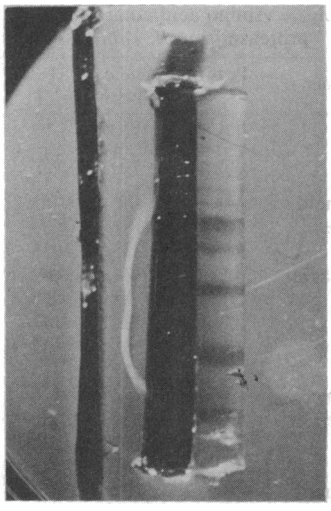

FIG. 3. Immunological cross-reactivity of multiple electrophoretic forms against bacillopeptidase F antiserum. Two 100-μg samples of pooled fractions of protease (peak A), eluted from a 7.5% polyacrylamide gel, were incubated for 2 h at 37°C in 0.05 M sodium carbonate buffer (pH 10) and electrophoresed in parallel (5, 20). The right-hand gel was stained for protein as described in the text. The R_f values for these proteins were 0.84, 0.63, 0.50, and 0.42; note that the lowermost band is the dye at the front. The other gel was embedded in immunodiffusion agar (dark band in the center). A 2-mm slit was cut in the gel (dark band at left) and filled with bacillopeptidase F antiserum. After 12 h at 22°C, white immunoprecipitin lines were clearly visible.

tidase F, we used the antiserum described above to detect cross-reacting fractions in Ouchterlony plates.

Three different preparations of bacillopeptidase F were made in the presence of PMSF at pH 6; only two peaks of cross-reacting material were ever detected under these conditions. Each of these proteins has been obtained in an electrophoretically pure state; they are referred to hereafter as peak 1 and peak 2. Peak 1 eluted first from the preparative-scale electrophoresis column. The two peaks gave identical precipitin bands in double-diffusion tests against bacillopeptidase F antiserum. Despite the addition of PMSF, some azocaseinase activity was detected for each of these enzymes after electrophoresis on polyacrylamide.

In one experiment, EDTA (final concentration, 2.5 mM [pH 7.0]) and PMSF (final concentration, 2.5 mM) were added to the growth medium just before the production of bacillopeptidase F (at 12 h; Fig. 1). Under these conditions, the cells produced amounts of cross-reacting material similar to amounts produced by untreated cultures. Purification of the enzyme yielded the same peak 1 and peak 2 proteins as were seen in the other preparations at pH 6.0. When isolations were done in the presence of PMSF, the faster-moving electrophoretic forms were never observed.

Attempts to reactivate bacillopeptidase F. Treatment of PMSF-inhibited peak 1 protein with carbonate buffer (pH 10), 0.13 M hydroxylamine (pH 7), or 0.5 M formhydroxymate (synthesized by the method of Uemitsu et al. [37]) at pH 8.5 caused no detectable reactivation of the enzyme. The fact that some protease activity was always detected after preparative gel electrophoresis suggests that conditions in the electrophoresis column promoted the hydrolysis of the sulfonate ester. We describe several properties of these two covalently inhibited proteins, obtained from several different preparations, below.

Estimation of molecular weights of peak 1 and peak 2. Molecular weights of each peak were determined by the meniscus depletion method of sedimentation equilibrium. The plots used for estimating molecular weight were linear. The second column of Table 2 shows the values obtained for peaks 1 and 2 by this method. Peak 1, which eluted from the preparative-scale electrophoresis column first, has a molecular weight similar to the value of 36,000 reported by Boyer and Carlton (2).

Molecular weights for each of the two peaks were also estimated by electrophoresis in SDS-urea polyacrylamide gels by the method of Swank and Munkres (33). The mobilities of standard proteins used for calibration gave linear standard curves. The molecular weights of

TABLE 2. Estimation of molecular weights for peaks 1 and 2 of bacillopeptidase F by equilibrium sedimentation and electrophoresis in SDS-urea gels

Peak	Sedimentation equilibrium mol wt (±SD)	SDS-urea mol wt (±SD)	
		Major protein	Minor protein
1	33,300[a] ±3,600	64,700[b] ±3,000	56,000[c] ±2,800
2	50,200[d] ±500	74,500[e] ±700	None present

[a] Average of six determinations from three separate purifications.

[b] Average of eight determinations from three separate purifications.

[c] Average of two determinations from three separate purifications; minor band only seen in one fraction.

[d] Average of two readings of the same photographic plate; a single determination from one purification.

[e] Average of two determinations from one purification.

the enzyme in peaks 1 and 2 were estimated by this method, and the values are summarized in the third column of Table 2.

Although the two methods used here did not give comparable values for the molecular weights of the two proteins, both methods showed that peak 2 was significantly larger than peak 1.

Testing for carbohydrate and lipid components. Because of the differences in the molecular weight values obtained by the two methods, we decided to test for nonprotein components. We tested for lipid as described in Materials and Methods (25); no lipid was detected. Tests for carbohydrate, however, were strongly positive (Fig. 4). Standard glycoproteins and proteins not conjugated to carbohydrate were analyzed in parallel; only glycoproteins became stained with basic fuchsin. Both peak 1 (Fig. 4) and peak 2 (not shown) proteins gave positive carbohydrate reactions. In addition to results shown for one isolation, peak 1 protein in three other isolations gave similar results.

Determination of amino acid compositions of peaks 1 and 2. As shown in Table 3, the amino acid composition of peak 1 is similar to that previously reported for the acidic protease of *B. subtilis*, estimated from its sedimentation rate to have a molecular weight of 36,000 (2). Some of the variations observed may be due to the fact, shown above, that this may be glycoprotein. Carbohydrate has been reported to interfere with amino acid recoveries (1). In addition, Boyer and Carlton (2) took no precautions to avoid autodigestion of their enzyme during isolation. On the other hand, the amino acid composition of peak 1 is distinctly different from that of peak 2; for example, the ratio of alanine to isoleucine was 3.4 for peak 1 but 1.2 for peak 2, and the ratios of acidic to basic amino acids are different (see below). Both proteins contain materials coeluted with phosphoserine and galactosamine. Phosphoserine has been identified in acid hydrolyzates of spore coat protein from *B. subtilis* (26), but it might not survive the hydrolysis conditions used here (23). Regardless of its chemical identity, both proteins were found to release the material on hydrolysis.

TABLE 3. Amino acid composition of serine protease from *B. subtilis* 168[a]

Constituent	Peak 1[b]	Acidic protease[c]	Peak 2[d]	ISP-Bsu 168[e]
Lysine	11	12	27	17
Histidine	7	5	13	10
Arginine	5	5	14	6
Aspartic acid	44	48	65	36
Threonine	18	18	33	13
Serine	26	25	35	23
Glutamic acid	44	33	49	38
Proline	20	25	18	13
Glycine	42[f]	43	44[f]	40
Alanine	38	38	41	26
Valine	20	21	25	19
Methionine	4	5	6	5–6
Isoleucine	11	14	35	13
Leucine	15	16	25	22
Tyrosine	6	9	26	7
Phenylalanine	8	10	11	8
Phosphoserine	2		3	

[a] All values are residues per mole.

[b] Based on a molecular weight of 33,300; average values of two separate hydrolyses.

[c] Data of Boyer and Carlton (2) corrected for loss of water from each residue and based on a molecular weight of 33,300.

[d] Based on a molecular weight of 50,200; average values of three separate hydrolyses.

[e] ISP-Bsu, Intracellular serine protease from *B. subtilis* 168 Marburg (31).

[f] Values were normalized from an observed molar ratio for Gly/Ala of 1.6 to the ratio of 1.13 reported by Boyer and Carlton (2).

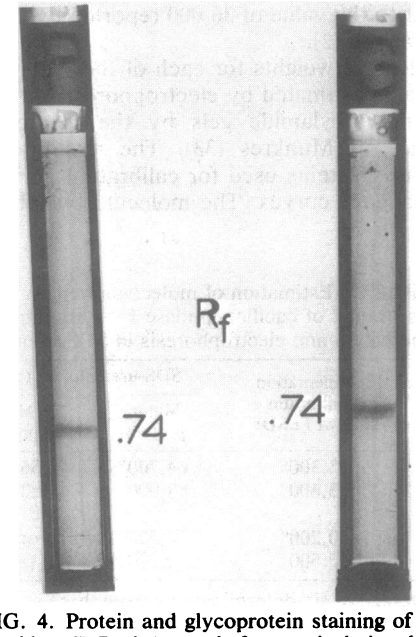

FIG. 4. Protein and glycoprotein staining of bacillopeptidase F. Peak 1 protein from an isolation done at pH 6.0 in the presence of PMSF was concentrated 10-fold and electrophoresed on 12.5% polyacrylamide gels (13 μg of protein per gel) by the preparative-scale electrophoresis procedure. One gel (P) was stained for protein with 0.25% (wt/vol) Coomassie blue G-250. The other gel (G) was treated with periodic acid and basic fuschin dye to analyze for carbohydrate by the method of Zacharius et al. (40) as outlined in the text.

Determination of isoelectric points. The isoelectric pH of the cross-reacting proteins in

peaks 1 and 2 was estimated by isoelectric focusing in polyacrylamide gels. The protein in peak 1 was found to have an isoelectric pH of 4.4, whereas that in peak 2 was found to be 5.4. Peak 1 has a ratio of acidic to basic amino acids of 5.5, which is similar to the value of 4.8 determined for the acidic protein of Boyer and Carlton (2) but quite different from the value of 2.8 calculated for peak 2. The intracellular serine protease of *B. subtilis* 168 isolated by Strongin et al. (31, 32) has a ratio of acidic to basic amino acids of 3.2. Intracellular and extracellular proteases have been compared by Stepanov et al. (30).

DISCUSSION

Three different research groups have reported observing multiple electrophoretic forms of bacillopeptidase F-like enzymes (11, 15, 23), but no one has yet proposed a source of this multiplicity of forms. Three observations presented here support the idea that some of the electrophoretic forms may have been produced by proteolysis: (i) bacillopeptidase F was shown to have strong proteolytic activity at pH 10; (ii) there was a time-dependent appearance of several enzymatically active electrophoretic forms; and (iii) when the enzyme was isolated in the presence of PMSF at pH 6 to minimize autodigestion, only two relatively high-molecular-weight forms were observed.

The immunological similarity of the multiple electrophoretic forms might be accounted for in two ways. First, the molecular weight of the enzyme used to raise the antibodies (Fig. 2) was not known (as it was used to prepare antiserum) and may itself have been a lower-molecular-weight fragment. Thus, its antigenic sites may have been present on the other electrophoretic forms detected. Alternatively, since the enzyme is a glycoprotein, the carbohydrate portion may have served as the major antigen and may not have been removed by the presumed proteolysis. In any case, it was possible to stabilize and characterize two higher-molecular-weight forms by covalently inhibiting the enzyme during isolation.

Both methods used to determine the molecular weights of the peaks 1 and 2 indicated that peak 2 was significantly larger than peak 1. The values obtained by the two methods, however, did not agree well with each other. Two explanations are offered. Glycoproteins not only are known to have altered shapes (8) but also are reported to bind SDS weakly (22) and to have altered electrophoretic mobilities (3, 27). In addition, proteins with low isoelectric points are also reported to bind SDS weakly. For these reasons, we believe the SDS-urea gel system we used may have given anomolous results, and the molecular weight values obtained by the sedimentation equilibrium method are closer to the true values. Voordouw et al. (38) found that equilibrium sedimentation methods were better than electrophoretic methods for estimating molecular weights of bacterial and fungal proteases. The molecular weight of peak 1 determined by equilibrium sedimentation (33,000 ± 3,500) is in good agreement with the value of 36,000 (sedimentation rate) reported for highly purified bacillopeptidase F by Boyer and Carlton (2) and 35,000 (SDS-gel electrophoresis) reported for an esterase fraction by Mäntsälä and Zalkin (16).

There are striking similarities between the two proteins. Both enzymes hydrolyze azocasein and are inhibited by PMSF; both enzymes appear to be glycoproteins, the first examples of bacterial glycoproteins of this type; both release, upon acid hydrolysis, compounds which comigrate with phosphoserine and galactosamine; and the two give identical immunoprecipitin patterns. Within experimental error, the amino acid composition data also show that peak 1 could be cleaved from the peak 2 protein; however, further experiments will be necessary before any conclusion can be drawn about the relationship of these two proteins.

In this regard, Strongin et al. (32) have reported that *B. subtilis* 1,000 M (a morphological mutant) produces an insoluble complex of protease containing 30,000- and 45,000-molecular-weight species (SDS-gel electrophoresis). They suggest that these high-mobility electrophoretic forms might be related to the intracellular serine protease (Table 3). It is not clear from their report whether the 45,000-molecular-weight species has any proteolytic activity. We believe that the amino acid compositions of intracellular serine protease of *B. subtilis* 168 and bacillopeptidase F are too different to consider them to be the same proteins. We suggest that the high-mobility electrophoretic forms reported by Strongin et al. may be forms of bacillopeptidase F.

Recently, Srivastava and Aronson (29) partially characterized a trypsin-like intracellular protease from *B. subtilis* JH 642 which was first observed by Hiroishi and Kadota (12). Aronson has found that the enzymatic activity of this trypsin-like protease was not inhibited by the antiserum we raised against bacillopeptidase F, whereas bacillopeptidase F activity was inhibited by these antibodies (A. Aronson, personal communication). This suggests that these two proteases are also distinct species.

ACKNOWLEDGMENTS

We thank Dennis Darnall for help in interpreting the sedimentation equilibrium data and Alex Kuroski and Thomas

Burnett for assistance with the amino acid analyses.

This work was supported by Public Health Service grant 5R01-GM 19643 from the National Institutes of Health.

LITERATURE CITED

1. **Blackburn, S.** 1968. Amino acid determination. Methods and techniques. Marcel Dekker, Inc., New York.
2. **Boyer, H. W., and R. C. Carlton.** 1968. Production of two proteolytic enzymes by a transformable strain of *Bacillus subtilis*. Arch. Biochem. Biophys. **128**:442–455.
3. **Bretscher, M. S.** 1971. Major human erythrocyte glycoprotein spans the cell membrane. Nature (London) New Biol. **231**:229–232.
4. **Chervenka, C. H.** 1973. A manual of methods for the analytical ultracentrifuge. Spinco Division of Beckman Instruments, Inc., Palo Alto, Calif.
5. **Davis, B. J.** 1964. Disc gel electrophoresis. II. Method and application to human serum protein. Ann. N.Y. Acad. Sci. **121**:404–427.
6. **Frehel, C., G. Dubray, and A. Wolff.** 1974. Activité estérasique des membranes cytoplasmiques et mésomiques au cours de la sporulation de *Bacillus subtilis*. Biochime **56**:583–598.
7. **Geele, G. E., E. Garrett, and J. H. Hageman.** 1975. The effect of benzeneboronic acids on sporulation and the production of serine protease in *Bacillus subtilis*, p. 391–396. *In* D. Gerhardt, R. N. Costilow, and H. L. Sadoff (ed.), Spores VI. American Society for Microbiology, Washington, D.C.
8. **Gibbons, R. A.** 1966. Physico-chemical methods for the determination of the purity, molecular size and shape of glycoproteins, p. 29–95. *In* A. Gottchalk (ed.), Glycoproteins, vol. 5, Elsevier Publishing Co., Amsterdam.
9. **Good, N. E., G. D. Winget, W. Winter, T. N. Connolly, S. Izawa, and R. M. M. Sing.** 1966. Hydrogen ion buffers for biological research. Biochemistry **5**:467–477.
10. **Hageman, J. H., and B. C. Carlton.** 1970. An enzymatic and immunological comparison of two proteases from a transformable *Bacillus subtilis* with the "subtilisins." Arch. Biochem. Biophys. **139**:67–79.
11. **Hageman, J. H., and B. C. Carlton.** 1973. Effects of mutational loss of specific intracellular proteases on the sporulation of *Bacillus subtilis*. J. Bacteriol. **114**:612–617.
12. **Hiroishi, S., and H. Kadota.** 1976. Intracellular proteases of *Bacillus subtilis*. Agric. Biol. Chem. **40**:1047–1049.
13. **Kalckar, H. M.** 1947. Differential spectrophotometry of purine compounds by means of specific enzymes. III. Studies of the enzymes of purine metabolism. J. Biol. Chem. **167**:461–475.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
15. **Mamas, S., and J. Millet.** 1975. Purification et propriétés d'une estérase excretée pendant la sporulation de *Bacillus subtilis*. Biochimie **57**:9–16.
16. **Mäntsälä, P., and H. Zalkin.** 1980. Extracellular and membrane-bound proteases from *Bacillus subtilis*. J. Bacteriol. **141**:493–501.
17. **Maurizi, M. R., and R. L. Switzer.** 1980. Proteolysis in bacterial sporulation. Curr. Top. Cell Regul. **16**:163–224.
18. **Millet, J.** 1969. Charactérisation de deux endopeptidases excrétées par *B. subtilis* Marburg au cours de la sporulaton. Bull. Soc. Chim. Biol. **51**:457–469.
19. **Millet, J.** 1970. Characterization of proteinases excreted by *Bacillus subtilis* Marburg strain during sporulation. J. Appl. Bacteriol. **23**:207–219.
20. **Ornstein, L.** 1964. Disc gel electrophoresis. I. Background and theory. Ann. N.Y. Acad. Sci. **121**:321–349.
21. **Ouchterlony, O. H.** 1968. Handbook of immunodiffusion and immunoelectrophoresis. Ann Arbor Science Publishers, Ann Arbor, Mich.
22. **Pitt-Rivers, R., and R. S. A. Impiobato.** 1968. Binding of sodium dodecyl sulfate to various proteins. Biochem. J. **109**:825–830.
23. **Plimmer, R. H. A.** 1941. Esters of phosphoric acid 4. Phosphoryl hydroxyamino acids. Biochem. J. **35**:461–469.
24. **Prestidge, L., V. Gage, and J. Spizizen.** 1971. Protease activities during the course of sporulation in *Bacillus subtilis*. J. Bacteriol. **107**:815–823.
25. **Ressler, N., R. Springgate, and J. Kaufman.** 1961. A method for lipoprotein electrophoresis. J. Chromatogr. **6**:409–415.
26. **Sano, K., T. Ichikawa, and M. Kondo.** 1975. Occurrence of phosphorylserine in the spore coat of *Bacillus subtilis* NRRL B558. Microbios **12**:67–76.
27. **Schubert, D.** 1970. Immunoglobulin biosynthesis. IV. Carbohydrate attachment to immunoglobulin subunits. J. Mol. Biol. **51**:287–301.
28. **Spizizen, J.** 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. U.S.A. **44**:1072–1078.
29. **Srivastava, O. P., and A. J. Aronson.** 1981. Isolation of a unique protease from sporulating cells of *Bacillus subtilis*. Arch. Microbiol. **129**:227–232.
30. **Stepanov, V. M., A. Y. Strongin, L. S. Isotova, Z. T. Abramov, L. A. Lyublinskaya, L. M. Ermakova, L. A. Baratova, and L. P. Belyanova.** 1977. Intracellular serine protease from *Bacillus subtilis*. Structural comparison with extracellular serine proteases—subtilisins. Biochem. Biophys. Res. Commun. **77**;298–305.
31. **Strongin, A. Y., D. I. Gorodetsky, I. A. Kuznetzova, V. V. Yanonis, Z. T. Abramov, L. P. Belyanova, L. A. Baratova, and V. M. Septanov.** 1979. Intracellular serine proteinase of *Bacillus subtilis* strain Marburg 168. Comparison with the homologous enzyme from *Bacillus subtilis* strain A-50. Biochem. J. **179**:333–339.
32. **Strongin, A. Y., L. I. Izotova, Z. T. Abramov, L. M. Ermakova, D. I. Gorodetsky, and V. M. Stepanov.** 1978. On the appearance of *Bacillus subtilis* intracellular serine protease in the cell membrane and culture medium. Arch. Microbiol. **119**:289–293.
33. **Swank, R. T., and K. D. Munkres.** 1971. Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. Anal. Biochem. **39**:462–477.
34. **Tsuru, D., H. Kira, T. Yamamoto, and J. Fukumoto.** 1966. Studies on bacterial protease. XIV. Zinc as an essential metal component of neutral protease of *Bacillus subtilis* var. *amylosacchariticus*. J. Agric. Biol. Chem. **30**:856–862.
35. **Tsuru, D., H. Kira, T. Yamamoto, and J. Fukumoto.** 1966. Studies on bacterial protease. XVI. Purification, crystallization and some enzymatic properties of alkaline protease of *Bacillus subtilis* var. *amylosacchariticus*. J. Agric. Biol. Chem. **30**:1261–1268.
36. **Tsuru, D., H. Kira, T. Yamamoto, and J. Fukumoto.** 1967. Studies on bacterial protease. XVII. Proteolytic specificity of neutral protease of *Bacillus subtilis* var. *amylosacchariticus*. J. Agric. Biol. Chem. **31**:718–723.
37. **Uemitsu, N., M. Sugiyama, and H. Matsumiya.** 1972. The reactivation of phenylmethanesulfonyl-subtilisin. Biochim. Biophys. Acta **258**:562–565.
38. **Voordouw, G., M. Gaucher, and R. S. Roche.** 1974. Anomalous molecular weights of proteases in gel chromatography. Biochem. Biophys. Res. Commun. **58**:8–12.
39. **Yphantis, D. A.** 1964. Equilibrium ultracentrifugation of dilute solutions. Biochemistry **3**:297–317.
40. **Zacharius, R. M., T. E. Zell, J. H. Morrison, and J. J. Woodlock.** 1969. Glycoprotein staining following electrophoresis on acrylamide gels. Anal. Biochem. **30**:148–152.