

Intracellular Serine Protease of *Bacillus subtilis*: Sequence Homology with Extracellular Subtilisins

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Intracellular serine protease was isolated from stationary-grown *Bacillus subtilis* A-50 cells and purified to homogeneity. The molecular weight of the enzyme is $31,000 \pm 1,000$, with an isoelectric point of 4.3. Its amino acid composition is characteristically enriched in glutamic acid content, differing from that of extracellular subtilisins. The enzyme is completely inhibited with phenylmethylsulfonyl fluoride and ethylenediaminetetraacetic acid. Intracellular protease possesses negligible activity towards bovine serum albumin and hemoglobin, but has 5- to 20-fold higher specific activity against *p*-nitroanilides of benzyloxycarbonyl tripeptides than subtilisin BPN'. Esterolytic activity of the enzyme is also higher than that of subtilisin BPN'. The enzyme is sequence homologous with secretory subtilisins throughout 50 determined NH₂-terminal residues, indicating the presence of duplicated structural genes for serine proteases in the *B. subtilis* genome. The occurrence of two homologous genes in the cell might accelerate the evolution of serine protease not only by the loosening of selective constraints, but also by creation of sequence variants by means of intragenic recombination. Three molecular forms of intracellular protease were found, two of them with NH₂-terminal glutamic acid and one minor form, three residues longer, with asparagine as NH₂ terminus. These data indicate the possible presence of an enzyme precursor proteolytically modified during cell growth.

A number of cell proteins are eliminated or transformed by dynamic processes of intracellular turnover or limited proteolysis (27). Proteolytic enzymes are implicated as agents of such conversions, but little is known about the specific functional role of individual proteases. The role of intracellular proteases might be especially significant for sporeforming *Bacilli* which undergo complex changes in physiology and morphology leading to the formation of dormant spores under nutrient deprivation (3). On physiological and genetic grounds it has been postulated that the appearance of proteolytic activity has to be somehow related to sporulation (19, 26, 29). Under normal conditions, sporulation coincides with an increased protein turnover, induction or derepression of new enzyme systems, modification of certain intracellular proteins, and excretion of a variety of enzymes (3, 8). Several intracellular as well as extracellular proteases may play an important role in these processes. In *Bacillus subtilis* grown in a liquid medium, three proteolytic enzymes are shown to be excreted during the sporulation phase, namely, alkaline serine protease (subtilisin), metalloprotease, and esterase, which possesses high esterolytic but low proteolytic activity (19, 26,

29). During the last decade, these enzymes, especially subtilisins, were extensively studied (17, 36). Nevertheless, the specific role of subtilisin in sporulation remains unclear, being still a matter of speculation (6, 8, 21). In contrast with subtilisin, the intracellular serine proteases are poorly characterized, although such enzymes have been detected in *B. subtilis* (9, 11, 30, 37) and related *Bacillus* species, *B. megaterium* (4, 20, 31), *B. cereus* (5), and *B. licheniformis* (1). None of the intracellular serine proteases of *B. subtilis* was isolated in a pure state in the amount suited for careful examination. Unfortunately, the lack of information concerning the relationship of intra- and extracellular serine proteases complicates their strict comparison and the interpretation of their biological functions in sporulation.

We succeeded in isolating pure intracellular serine protease (ISP) from stationary-grown *B. subtilis* A-50. In this paper we will describe the procedure of this protease purification along with its functional and structural characteristics. The location of the enzyme in the cell and the conditions for its appearance outside of the cell, as well as detailed comparison of our data on ISP with those reported earlier, will be the sub-

ject of another communication. The preliminary results of our study were reported previously (34).

MATERIALS AND METHODS

Bacterial strain. *B. subtilis* A-50, used throughout this study, was a gift of L. Keay (New Enterprise Division, Monsanto Company, St. Louis, Mo.).

Materials. DEAE-cellulose DE-52 was purchased from Whatman, Sephadex G-100 and Sepharose 4B from Pharmacia, and Ultrogel AcA-34 from LKB. Benzoyloxycarbonyl-Gly-Gly-L-Leu-*p*-nitroanilide (Z-Gly-Gly-L-Leu-*p*NA), benzoyloxycarbonyl-Gly-Gly-L-Phe-*p*-nitroanilide (Z-Gly-Gly-L-Phe-*p*NA), and benzoyloxycarbonyl-L-Ala-L-Ala-L-Leu-*p*-nitroanilide (Z-L-Ala-L-Ala-L-Leu-*p*NA) were synthesized in our laboratory by L. A. Lyublinskaya as described earlier (15, 16).

Tris(hydroxymethyl)aminomethane (Tris) from Sigma; *p*-hydroxymercuribenzoate, sodium dodecyl sulfate (SDS), urea, CNBr, phenylmethylsulfonyl fluoride (PMSF), acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, sucrose, ethylenediaminetetraacetic acid, ethyleneglycol-bis(β -aminoethyl ether)-*N,N*-tetraacetic acid, riboflavin, 2-mercaptoethanol, and Coomassie brilliant blue R-250 from Serva; Cyanogum 41 (acrylamide-methylenebisacrylamide, ratio 19:1) from Merck; tosyl-L-arginine methyl ester, benzoyl-L-arginine ethyl ester, and benzoyl-L-tyrosine methyl ester from Reanal; and acetyl-L-tyrosine ethyl ester and benzoyl-L-tyrosine ethyl ester from Fluka, as well as all other reagents used, were of analytic grade.

Myoglobin, chymotrypsinogen, ovalbumin, and bovine serum albumin were from Serva. Porcine pepsin was purified in our laboratory.

Extracellular serine protease from *B. subtilis* (subtilisin) was obtained from Serva (Subtilopeptidase A) or Nagase (Nagase) and isolated from *B. subtilis* A-50 (subtilisin A-50) in our laboratory as described earlier (35). All these subtilisins belong to the subtilisin type BPN' (synonym Novo) as judged by amino acid analysis (17).

Protease assay. Incubation mixture contained 0.005 to 0.1 ml of enzyme solution, 50 mM Tris-1 mM CaCl₂ (pH 8.5) up to 1.25 ml, and 0.25 ml of Z-L-Ala-L-Ala-L-Leu-*p*NA in dimethylformamide (0.5 mg/ml). After 5 to 15 min of incubation at 40°C, the reaction was stopped with 0.5 ml of 2 M sodium citrate buffer (pH 5.0), and the amount of *p*-nitroaniline released was measured at 410 nm (15). The molar absorbance of *p*-nitroaniline at 410 nm was equal to 8,900 M⁻¹ cm⁻¹. Z-Gly-Gly-L-Leu-*p*NA and Z-Gly-Gly-L-Phe-*p*NA were also used under the same conditions from stock solutions of 1 mg/ml and 0.5 mg/ml, respectively. One unit of activity is equal to the amount of the enzyme that hydrolyzes 1 μ mol of the substrate per min under the standard conditions.

Proteolytic activity was determined with hemoglobin and bovine serum albumin as substrates. The reaction was carried out in a mixture containing 0.1 ml of enzyme solution, 50 mM Tris-1 mM CaCl₂ (pH 8.5) up to 1 ml, and 1 ml of substrate. Substrates were dissolved in 50 mM Tris-1 mM CaCl₂ (pH 8.5) to a

final concentration of 2.5%. After 15 to 30 min at 40°C, the reaction was stopped with 5 ml of 5% trichloroacetic acid. The insoluble material was removed by filtration, and the absorbance of the filtrate was measured with a spectrophotometer at 280 nm.

Esterolytic activity assay. Esterolytic activity was measured at 20°C in a Gilford 240 recording spectrophotometer with a reference cuvette containing all components of the assay except enzyme. Benzoyl-L-arginine ethyl ester, benzoyl-L-tyrosine methyl ester, tosyl-L-arginine methyl ester, and acetyl-L-tyrosine ethyl ester were used as substrates. Hydrolysis of the first four esters was monitored by an increase in optical density at 254 nm and reaction of the last one by a decrease in optical density at 237 nm (29). The reaction mixture contained 1.7 ml of the enzyme solution in 50 mM Tris-1 mM CaCl₂ (pH 8.5), 0.3 ml of 1 M glycine-NaOH buffer (pH 9.1), and 1 ml of the substrate.

Protein assay. Protein concentration was measured by absorbance at 280 nm or by the Lowry method (14) using bovine serum albumin as a standard.

Thin-layer gel isoelectrofocusing. Gel isoelectrofocusing was performed at 4°C using an LKB Multiphor apparatus and LKB Ampholine PAG plates (ampholine range, pH 3.5 to 9.5) as described by the manufacturer. After focusing, the gels were stained with Coomassie brilliant blue R-250 (39). pH gradient was determined at 20°C after soaking gel slices in 1 ml of deionized water.

Electrophoresis. SDS-polyacrylamide gel electrophoresis was performed as described by Weber and Osborn (40) using 10% acrylamide gels in 0.1 M sodium phosphate buffer (pH 7.1) containing 0.1% SDS. Before electrophoresis, protease samples were treated with 1 mM PMSF for 60 min at 20°C, followed by heating in 10 mM sodium phosphate buffer (pH 7.1) containing 1% SDS and 1% 2-mercaptoethanol for 2 to 3 min at 100°C. For molecular weight estimation, myoglobin (molecular weight 17,800), chymotrypsinogen (25,000), subtilisin BPN' (28,000), pepsin (35,000), ovalbumin (45,000), and bovine serum albumin (67,000), were used as the references. Bromophenol blue was the tracking dye.

Polyacrylamide gel electrophoresis under non-denaturing conditions was carried out at 5°C as described by Davis (7), using 10% or 7.5% Cyanogum 41 gels. *R_f* of the protein bands was calculated against bromophenol blue. To localize the active bands, 1.5-mm slices of the gel were incubated in 0.5 ml of 50 mM Tris-1 mM CaCl₂ (pH 8.5) containing 0.1 mg of Z-L-Ala-L-Ala-L-Leu-*p*NA per ml for several hours at 40°C, then *p*-nitroaniline released was estimated at 410 nm.

Gel filtration. For molecular weight estimation, gel filtration on a column (1.5 by 100 cm) of Sephadex G-100 superfine was performed (2). Buffers used were 50 mM sodium citrate-100 mM NaCl (pH 5.7), 50 mM sodium citrate-1 M NaCl (pH 5.7), 50 mM sodium citrate-100 mM NaCl-1% 2-mercaptoethanol (pH 5.7), 50 mM sodium acetate-100 mM NaCl (pH 4.7), 50 mM Tris-100 mM NaCl-1% 2-mercaptoethanol (pH 8.5), or 50 mM Tris-1 M NaCl (pH 8.5), each containing 1 mM CaCl₂. Bovine serum albumin, ovalbumin, pepsin, chymotrypsinogen, and myoglobin were run as

standards. Protease peak was located by activity against Z-L-Ala-L-Ala-L-Leu-pNA. The column void volume was determined by using a blue dextran standard.

CNBr treatment. Inactivated ISP (1 mg) was dissolved in 70% trifluoroacetic acid, and 2 mg of CNBr was added; the resulting solution was then incubated at 20°C for 24 h. The solution was diluted with water and evaporated in vacuo. The residue was dissolved in 8 M urea-10 mM Tris (pH 9.0) and subjected to polyacrylamide gel electrophoresis in 6 M urea-10% Cyanogum 41 gels according to Davis (7). The gels were stained with 0.3% Coomassie brilliant blue R-250 in 30% trichloroacetic acid and destained in 7.5% acetic acid. For comparison, subtilisin BPN' was treated and analyzed in parallel.

Amino acid analysis. The samples were hydrolyzed in 5.7 N HCl at 110°C in vacuo for 24 or 72 h, evaporated, and analyzed on a Durrum D-500 amino acid analyzer. Half-cysteine and methionine were determined as cysteic acid and methionine sulfone after performic acid oxidation (12).

Automated sequential Edman degradation. Automated Edman degradation was performed on a Beckman 890 sequencer equipped with a Beckman 890C reaction cup. Modified protein program no. 050972 with 0.2 M Quadrol was used at 60 to 62°C. As a rule, the protein amount was 50 to 200 nmol. *n*-Chlorobutane contained 0.1 mM dithiothreitol (10). Thiazolinones of amino acids were converted into corresponding phenylthiohydantoin under the standard conditions. For the identification of phenylthiohydantoin or their respective trimethylsilyl derivatives, ethylacetate and, when necessary, water layers were analyzed by gas chromatography (28) and thin-layer chromatography on silica gel plates. The identification of some residues was confirmed by hydrolysis of the respective derivatives with 5.7 N HCl in the presence of 0.1% SnCl₂ (18) at 150°C for 4 h with subsequent amino acid analysis.

Preparation of Gramicidin S-Sepharose 4B. Sepharose 4B (300 ml) was suspended in 450 ml of 5 M potassium phosphate buffer (pH 11.8) and activated by addition of 25 g of CNBr dissolved in 15 ml of acetonitrile. After 10 min at 7°C, the activated Sepharose was filtered and washed with iced 0.1 M NaHCO₃, then resuspended in the same solution; 7 g of twice-crystallized commercial Gramicidin S, dissolved in 210 ml of dimethylformamide plus 70 ml of 0.1 M NaHCO₃, was added (33). The mixture was gently stirred for 20 h at 20°C and then washed repeatedly with a large volume of 50% dimethylformamide and water and equilibrated with 50 mM Tris-1 mM CaCl₂ (pH 8.5). As judged by amino acid analysis, the sorbent contained about 1 μmol of Gramicidin S per ml of wet gel.

Cell growth and protease purification. Fermentation flasks (500-ml) containing rich nutrient Hottinger medium were inoculated from an agar plate culture of *B. subtilis* A-50 and incubated overnight at 37°C on a rotary shaker (200 rpm). This culture was used to inoculate 15 liters of modified Spizizen medium (32) in a 20-liter fermentor or 75 liters of the same medium in a 100-liter fermentor. After 20 to 22 h at 35°C, the cells were harvested by centrifugation, washed with

iced 50 mM sodium phosphate-100 mM NaCl (pH 6.5), and kept frozen at -20°C. About 300 g of the cells (wet weight) was used for enzyme purification.

(i) Extraction of cells. The cells were thawed, suspended in iced 50 mM Tris-1 mM CaCl₂ (pH 8.5), and disrupted by sonic treatment of small portions, each for 15 min at 4°C, with an MSE sonifier. All purification steps were performed at 4°C. The mixture was centrifuged at 30,000 × *g* for 60 min. The pellet was resuspended in the same buffer, and centrifugation was repeated. The supernatants from these procedures were collected and termed "crude extract."

(ii) Streptomycin sulfate precipitation. To the crude extract, streptomycin sulfate was added to a final concentration of 1 mg/ml. After 2 h at 0°C, the precipitated nucleic acids were removed by centrifugation at 10,000 × *g* for 30 min.

(iii) Ammonium sulfate fractionation. The supernatant from the last step was brought to 55% saturation with solid ammonium sulfate. The precipitated proteins containing no activity were discarded after centrifugation, and the supernatant was brought to 80% saturation by addition of solid ammonium sulfate. The precipitate containing enzyme activity was collected by centrifugation, dissolved in 50 mM Tris-1 mM CaCl₂ (pH 8.5), and dialyzed overnight against a large volume of the same buffer.

The enzyme was further purified by DEAE-cellulose DE-52 and Ultrogel AcA-34 chromatography, followed by polyacrylamide gel electrophoresis as described earlier (34). Alternatively, affinity chromatography was used omitting DEAE-cellulose and Ultrogel steps.

(iv) Affinity chromatography on Gramicidin S-Sepharose 4B. The dialyzed material from step iii was applied to a Gramicidin S-Sepharose 4B column (2 by 35 cm) equilibrated with 50 mM Tris-1 mM CaCl₂ (pH 8.5). Contaminating proteins were removed from the column by washing with the same buffer until the optical density at 280 nm reached zero, then by additional washing with 50 mM Tris-1 mM CaCl₂-1 M NaCl (pH 8.5). Protease fraction tightly bound to the sorbent was eluted with 50 mM Tris-1 mM CaCl₂-1 M NaCl (pH 8.5) containing 25% isopropanol. Active fractions were pooled, dialyzed against 50 mM Tris-1 mM CaCl₂ (pH 8.5), and subjected to re-chromatography under the same conditions. After re-chromatography, pooled and dialyzed active fractions were stored at 4°C and used for enzymological tests.

Separation of protease molecular forms (ISP-A and ISP-B) by polyacrylamide gel electrophoresis. To prevent autolysis, protease samples were completely inhibited with 1 mM PMSF for 60 min at 20°C and subjected to polyacrylamide gel electrophoresis under nondenaturing conditions as described above. For these runs, tubes (6 by 100 mm) with 10% Cyanogum 41 gels loaded with 1 mg of protein sample were used. After the completion of electrophoresis, the gel slices containing enzyme bands (the location of protease bands was visualized in a parallel gel stained with Coomassie blue for a few moments) were collected and homogenized with a glass homogenizer, and the protein was extracted overnight in 50 mM Tris-1 mM CaCl₂-100 mM NaCl (pH 8.5). Gel particles were removed by filtration through a sintered glass filter

no. 4 and by centrifugation at $18,000 \times g$ for 30 min. The supernatant was dialyzed thoroughly against water and freeze dried. Then the samples were denatured with phenol-water mixture (10:1 vol/vol), precipitated and washed with acetone and ether, and dried in vacuo. After such procedure, the protease samples were used for amino acid analysis, CNBr treatment, and structural investigations.

RESULTS

Identification of intracellular protease activity. When exponentially grown *B. subtilis* A-50 cells were examined for their content of ISP, only negligible activity was detected. On the contrary, stationary-grown cells showed substantial level of activity, about 20 to 30% as compared with the total activity of excreted subtilisin. When analyzed electrophoretically in 7.5% acrylamide or 10% acrylamide gels, two active intracellular protein fractions with R_f 0.6 (minor form) and R_f 0.7 (major form), or R_f 0.4 (minor form) and R_f 0.5 (major one), respectively, were revealed in the crude extract (Fig. 1); these differed markedly from the R_f of extracellular subtilisins, which were 0.08 (major molecular form), 0.16, and 0.3 (two minor forms) in 7.5% gels (35). The bulk of intracellular activity (80 to 90%) was present in the soluble fraction of the cell extract. About 10 to 20% of the total

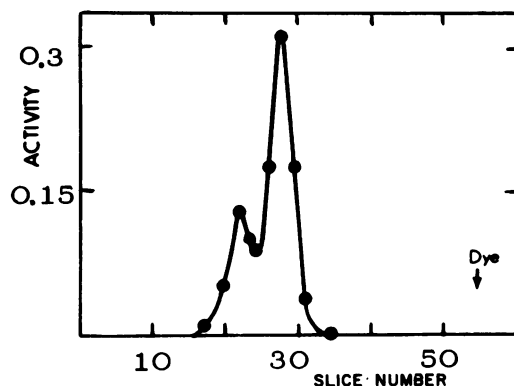


FIG. 1. Measurement of ISP activity in gel slices after polyacrylamide gel electrophoresis of the crude cell extract of *B. subtilis* A-50 cells. Gel concentration 10%.

intracellular activity was membrane bound and might be solubilized with detergent treatment (manuscript in preparation). Therefore, the stationary-grown *B. subtilis* A-50 cells were used as a source of intracellular serine protease.

Purification of ISP. The purification procedure (Table 1) detailed in Materials and Methods consisted of cell disruption by sonic treatment (step i), removal of nucleic acids by streptomycin sulfate precipitation (step ii), fractionation with solid ammonium sulfate (step iii), and affinity chromatography and re-chromatography on a Gramicidin S-Sepharose column (Fig. 2; step iv). After the last step, the yield of activity was about 50%, and the enzyme samples showed a 400-fold increase in specific activity over the step ii preparation, being pure as determined by polyacrylamide gel electrophoresis (Fig. 3I). Such ISP preparations were used for the enzymological studies.

Evidence of homogeneity. The homogeneity of the isolated active ISP preparations was checked by polyacrylamide gel electrophoresis. Only two protein bands, both possessing enzyme activity, were visualized. These active bands, corresponding to the enzyme forms found in the crude extract, were termed ISP-A and ISP-B. The minor form, ISP-B, had an R_f of 0.6 or 0.4 when studied in 7.5% or 10% gels, respectively. The major form of the enzyme, ISP-A, had R_f 0.7 or 0.5 in 7.5% or 10% gels, respectively (Fig. 3I).

Separation of ISP-A and ISP-B. For structural investigation, ISP forms were separated by polyacrylamide gel electrophoresis. Prior to electrophoresis, ISP was inhibited with PMSF to prevent autolysis, since electrophoresis of non-inhibited samples led to a two- to threefold decrease of specific activity. Inhibited ISP samples recovered from gels were electrophoretically pure and well suited for structural analysis. By the method employed ISP-A and ISP-B were quantitatively separated (Fig. 3).

Electrophoretic behavior of ISP and subtilisin BPN'. Electrophoretic behavior of ISP-A and subtilisin BPN' (subtilisin A-50) previously isolated from the same strain of *B. subtilis* A-50 (35) is shown in Fig. 4. As described earlier

TABLE 1. Purification of ISP from sporulating *B. subtilis* A-50

Step	Protein (mg)	Total activity (U)	Sp act ^a	Purification (fold)	Yield (%)
i. Crude extract (30,000 × g)		2,800			100
ii. Streptomycin sulfate	65,500	3,100	0.048	1	110
iii. Ammonium sulfate (55 to 80%)	3,000	2,250	0.75	15.5	80
iv. Affinity chromatography	80	1,450	18.1	380	52

^a Micromoles of *p*-nitroaniline released per milligram of protein per minute. Z-L-Ala-L-Ala-L-Leu-pNA was used as substrate.

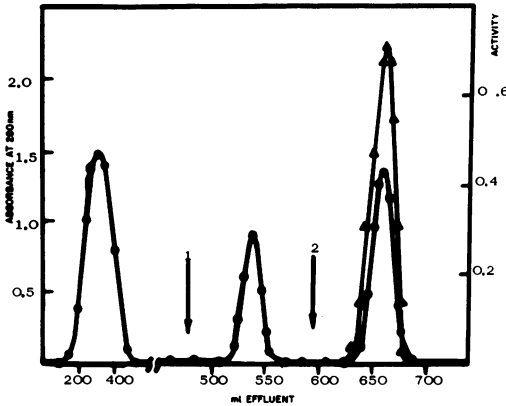


FIG. 2. Affinity chromatography of partially purified ISP on Gramicidin S-Sepharose 4B. The column (2 by 35 cm) was equilibrated with 50 mM Tris-1 mM CaCl_2 (pH 8.5). Contaminating proteins were eluted with the same buffer and 50 mM Tris-1 mM CaCl_2 -1 M NaCl, pH 8.5 (the first arrow). ISP was eluted with 50 mM Tris-1 mM CaCl_2 -1 M NaCl-25% isopropanol, pH 8.5 (the second arrow). Symbols: ●, absorbance at 280 nm; ▲, ISP activity.

(35), subtilisin A-50, as well as other subtilisins of BPN' type, consisted of three active molecular forms with R_f 0.08, 0.16, and 0.3 (pI 8.15, 7.6, and 7.0, respectively). The first two forms of subtilisin A-50 are visible on the given photograph, and their R_f clearly differs from that of ISP-A.

Isoelectric point. The isoelectric point of ISP-A was determined with thin-layer gel isoelectrofocusing, using Ampholine pH range 3.5 to 9.5. When PMSF-inhibited enzyme was subjected to isoelectrofocusing, only one band with pI 4.3 appeared on the gel. This value is much lower than that of subtilisin BPN' molecular forms (35). In contrast, numerous stained bands with pI within a wide pH range were visible on the gel after the separation of noninhibited enzyme, presumably due to autolysis during isoelectrofocusing enhanced by Ca^{2+} sequestering. Pronounced autolysis during isoelectrofocusing was observed earlier for extracellular noninhibited subtilisin BPN' preparations (35).

Molecular weight. The molecular weight of ISP-A and ISP-B as estimated by SDS-polyacrylamide gel electrophoresis was $31,000 \pm 1,000$. These ISP forms migrated as one band upon SDS-electrophoresis, having, presumably, identical or very close molecular weights. As it appears in Fig. 4III, the molecular weight of ISP (upper band) is higher than that of subtilisin BPN' (lower faint band). Nevertheless, it should be mentioned that the molecular weight of ISP might be slightly overestimated due to the eventual anomalies in SDS binding by a rather acidic enzyme.

On the other hand, the molecular weight of nondenatured active ISP estimated by gel filtration on a Sephadex G-100 column was 54,000 to 56,000. This value did not change in the solutions of high ionic strength (up to 1 M NaCl) at pH 4.7 to 8.5 or in the presence of 1% 2-mercaptoethanol. Therefore, presumably, the active ISP exists in solutions as a dimer (cf. reference 25).

Stability, pH and temperature dependence, and spectra. When kept in 50 mM Tris-1 mM CaCl_2 (pH 8.5) at 4°C, the enzyme appeared stable and could be stored for at least 2 months without any detectable loss of activity. Without Ca^{2+} , the enzyme lost activity very rapidly and irreversibly at 20°C or even at 4°C.

The pH optimum of the enzyme activity against Z-L-Ala-L-Ala-L-Leu-pNA lies in the alkaline region, showing a plateau between pH 7.5 and 10.0 (Fig. 5). ISP was inactivated at pH below 4.5, but was rather stable in the alkaline

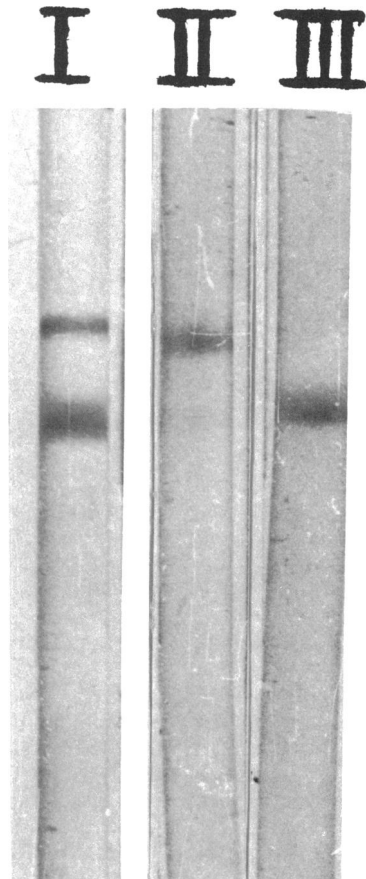


FIG. 3. Polyacrylamide gel electrophoresis of purified ISP. (I) ISP preparation after affinity chromatography; (II) ISP-B; (III) ISP-A. Gel concentration 10%. Migration from top to bottom. ISP-A and ISP-B were separated as described in the text.

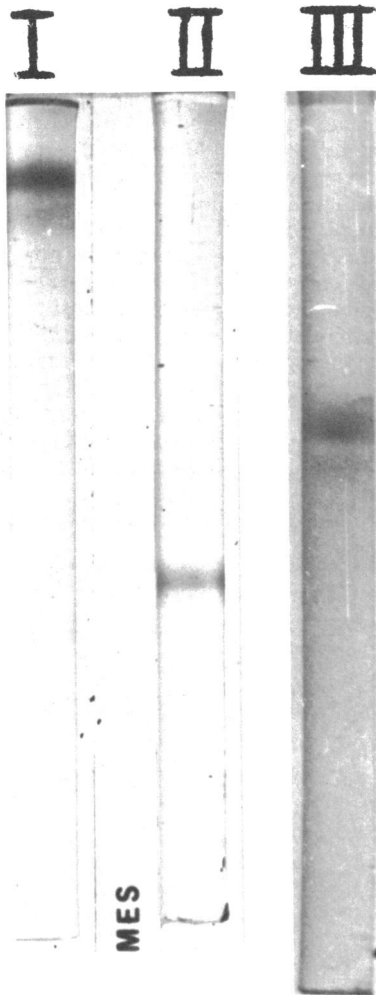


FIG. 4. Electrophoretic comparison of ISP-A and subtilisin BPN'. (I) Polyacrylamide gel electrophoresis of subtilisin BPN' and (II) of ISP-A in 7.5% gels. (III) SDS-polyacrylamide gel electrophoresis of ISP-A (upper band) and subtilisin BPN' (lower faint band). Migration from top to bottom.

pH range. Optimum for enzyme stability was pH 6.5 to 7.0.

In the presence of 1 mM Ca^{2+} , no loss of activity was detected in 60 min at 60°C, but heating for 30 min at 70°C led to significant inactivation of the enzyme. Without Ca^{2+} , the enzyme activity was lost irreversibly at 20°C in 30 min. It is of interest that, being stable in the presence of Ca^{2+} up to 60°C, ISP revealed maximal activity for Z-L-Ala-L-Ala-L-Leu-pNA hydrolysis at 40°C.

The enzyme possessed a typical protein absorption spectrum with a maximal absorption at 280 nm. Fluorescence emission spectrum of ISP

was practically the same as that of subtilisin BPN' with the maximum at 360 nm.

Effect of inhibitors and metal ions. PMSF, the inhibitor of serine proteases, at 1 mM inactivates the enzyme completely. Hence, intracellular protease from *B. subtilis* may be classified as a serine protease. *p*-Hydroxymercuribenzoate in the concentration range 10^{-4} to 10^{-3} M led to a weak inhibition of the enzyme; only 20% of activity was lost. Ethylenediaminetetraacetic acid and ethyleneglycol-bis(β -aminoethyl ether)-*N,N*-tetraacetic acid, chelating agents for Ca^{2+} ions, at 2 mM completely and irreversibly inhibited ISP. After this treatment, the activity could not be restored by the addition of divalent ions tested: Ca^{2+} , Zn^{2+} , Co^{2+} , Mn^{2+} , or Mg^{2+} . It should be mentioned that Ca^{2+} omission at any purification step led to rapid and irreversible inactivation of the enzyme, and enzyme activity could not be restored by further addition of Ca^{2+} . It seems likely that Ca^{2+} ions are absolutely required for ISP stability.

Substrate specificity. ISP cleaves subtilisin chromogenic substrates, *p*-nitroanilides of benzoyloxycarbonyl tripeptides, Z-L-Ala-L-Ala-L-Leu-pNA, Z-Gly-Gly-L-Phe-pNA, and Z-Gly-Gly-L-Leu-pNA, the first being the most suitable (Table 2). ISP preparations had a 5- to 20-fold higher specific activity against these substrates than did commercial BPN' subtilisins from Nagase or Serva or subtilisin A-50. On the contrary, when hemoglobin or bovine serum albumin was used as substrate, low activity was detected, although subtilisin BPN' cleaves these protein substrates rapidly.

ISP has a rather high esterolytic activity,

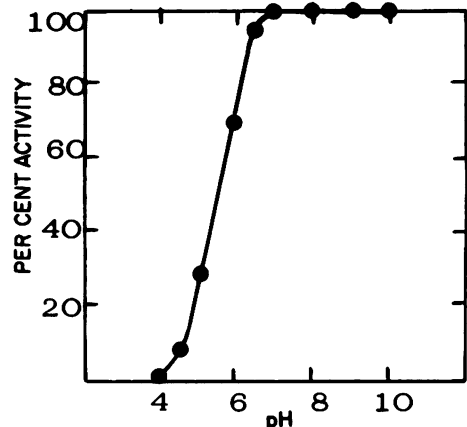


FIG. 5. Effect of pH on the activity of purified ISP. pH 4.0 to 6.2, 50 mM sodium citrate-1 mM CaCl_2 ; pH 7.0 to 10.0, 50 mM Tris-1 mM CaCl_2 . Activity is expressed as percentage of the maximal rate of hydrolysis.

TABLE 2. *Relative activity of ISP and subtilisin BPN'*

Substrate	Activity	
	ISP	Subtilisin BPN'
Z-L-Ala-L-Ala-L-Leu-pNA ^a	18.1	0.9
Z-Gly-Gly-L-Phe-pNA ^a	0.45	0.060
Z-Gly-Gly-L-Leu-pNA ^a	0.72	0.065
Benzoyl-L-arginine ethyl ester ^b	0.155	0.035
Benzoyl-L-tyrosine ethyl ester ^b	2.1	1.5
Benzoyl-L-tyrosine methyl ester ^b	2.0	2.0
Acetyl-L-tyrosine ethyl ester ^b	3.1	1.4

^a Specific activity. The conversion of chromogenic substrates was 5 to 10% (15, 16).

^b Esterolytic activity is expressed in arbitrary units, not comparable with those for chromogenic peptide substrates.

cleaving such ester substrates as benzoyl-L-arginine ethyl ester, benzoyl-L-tyrosine methyl and ethyl esters, and acetyl-L-tyrosine ethyl ester, but does not hydrolyze tosyl-L-arginine methyl ester. In comparison with subtilisin BPN', ISP specific activity against benzoyl-L-arginine ethyl ester and acetyl-L-tyrosine ethyl ester is 5- and 2.2-fold higher, respectively (Table 2).

Amino acid composition. The amino acid composition of ISP was reported earlier (34). ISP-A and ISP-B forms have the same amino acid composition within an experimental error. The enzyme has a characteristically high content of Glx, Asx, and Lys residues. In addition to the results reported in our previous work, we now have determined the presence of one residue of cysteic acid per molecule of performic acid-oxidized ISP.

CNBr peptides. Peptide fragments, obtained after the treatment of ISP-A with CNBr, were separated electrophoretically in 6 M urea-10% Cyanogum 41 gels. Subtilisin BPN' was treated with CNBr and analyzed in parallel. The electrophoretic pattern of CNBr peptides of ISP-A (five to six main fractions) and subtilisin BPN' (four main fractions) differed markedly, indicating a difference in the location and number of Met residues and/or in the composition of the peptide fragments (Fig. 6).

NH₂-terminal sequence determination. Electrophoretically pure phenol-denatured ISP-A samples were subjected to the automated sequential Edman degradation. The sequence of 19 NH₂-terminal amino acid residues was determined in the first run (34) and through 50 residues in the second run. The polypeptide chain

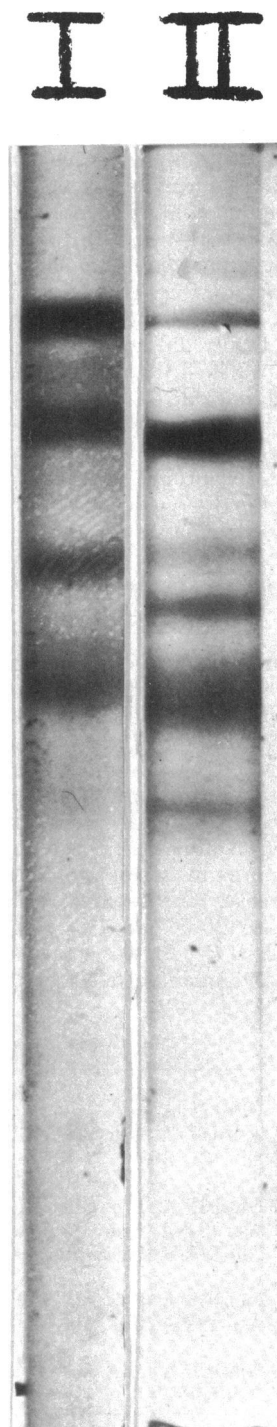


FIG. 6. Polyacrylamide gel electrophoresis of CNBr cleavage products of subtilisin BPN' (I) and ISP-A (II). 10% polyacrylamide gels contained 6 M urea. Migration from top to bottom.

NH₂-terminal sequences of ISP-A and ISP-B were determined by the automated Edman degradation. ISP sequence has been traced for 50 amino acid residues, although some residues remote from the NH₂ terminus of the enzyme could not be identified unambiguously (Table 3). The comparison of this sequence with those of secretory subtilisins clearly shows that ISP is homologous to them (Table 4). For 46 amino acid residues compared, 24 were identical with those in subtilisin BPN', 26 with subtilisin Amylosacchariticus, and 29 with subtilisin Carlsberg. It should be stressed that *B. subtilis* A-50 produces secretory subtilisin, which belongs to BPN' type as confirmed by a special run of sequence determination. Thus, it is possible to compare NH₂-terminal sequences of subtilisin A-50 and ISP, two serine proteases from the same strain, *B. subtilis* A-50. The minimal number of amino acid substitutions for ISP versus subtilisin BPN', as well as subtilisin A-50, is 22; for ISP versus subtilisin Amylosacchariticus the minimal number is 20; and for ISP versus subtilisin Carlsberg, the minimal number is 17.

These numbers are very close to 20 substitutions for the same sequence positions between Carlsberg and BPN' subtilisins or to 17 substitutions between Carlsberg and Amylosacchariticus subtilisins. The residues surrounding Asp-32 (Asp-30 of subtilisins, which belongs to their active site) are strictly conserved in ISP and subtilisins. Hence, the extent of NH₂-terminal sequence divergence is practically the same for intracellular as well as extracellular serine proteases. We assume that these enzymes evolved from the same ancestral serine protease. Nevertheless, the very specific shift in amino acid composition of ISP (its enrichment in Glx, Asx, and Lys) allows us to consider ISP as a new type of subtilisin-like protease rather than just an additional member of the known family of secretory subtilisins.

A very characteristic pattern of ISP NH₂-terminal sequence has to be mentioned. In the stretch of 23 residues, each fourth residue is Glu, Gln, Asp, or Lys. Taking in account the presence of two short α helices in this part of the subtilisin molecule (13, 17), one can assume that the side

TABLE 4. Alignment of NH₂-terminal sequences of ISP and secretory subtilisins^a

BPN'	Ala	Gln	Ser	Val	Pro	Tyr	Gly	Val	Ser	Gln	Ile	Lys	Ala	Pro
CAR	Ala	Gln	Thr	Val	Pro	Tyr	Gly	Ile	Pro	Leu	Ile	Lys	Ala	Asp
AMY	Ala	Gln	Ser	Val	Pro	Tyr	Gly	Ile	Ser	Gln	Ile	Lys	Ala	Pro
ISP			¹ Glu	Leu	Pro	Glu	Gly	Ile	Gln	Val	Ile	¹⁰ Lys	Ala	Pro
BPN'	Ala	Leu	His	Ser	Gln	Gly	Tyr	Thr	Gly	Ser	Asn	Val	Lys	Val
CAR	Lys	Val	Gln	Ala	Gln	Gly	Phe	Lys	Gly	Ala	Asn	Val	Lys	Val
AMY	Ala	Leu	His	Ser	Gln	Gly	Tyr	Thr	Gly	Ser	Asn	Val	Lys	Val
ISP	Gln	Leu	Trp	Ala	Gln	Gly	Phe	²⁰ Lys	Gly	Ser	Asp	Ile	Lys	Ile
BPN'	Ala	Val	Ile	Asp	Ser	Gly	Ile	Asp	Ser	Ser	His	Pro	Asn	Leu
CAR	Ala	Val	Leu	Asp	Thr	Gly	Ile	Gln	Ala	Ser	His	Pro	Asn	Leu
AMY	Ala	Val	Ile	Asp	Ser	Gly	Ile	Asp	Ser	Ser	His	Pro	Asn	Leu
ISP	Ala	Val	Leu	³⁰ Asp ^b	Thr	Gly	Ile	Asp	Val	X	X	Pro	⁴⁰ Asn	Leu
BPN'	Lys	Val	Ala	Gly	Gly	Ala	Ser	Met	Val	Pro	Ser			
CAR	Asn	Val	Val	Gly	Gly	Ala	Ser	Phe	Val	Ala	Gly			
AMY	Asn	Val	Arg	Gly	Gly	Ala	Ser	Phe	Val	Pro	Ser			
ISP	Asp	X	X	Gly	Gly	— ^c	X	Phe	Val	Ala	⁵⁰ Gly			

^a NH₂-terminal sequences of secretory subtilisins BPN' from *B. amyloliquefaciens* (BPN), Carlsberg from *B. subtilis* (CAR), and Amylosacchariticus from *B. amylosacchariticus* (AMY) are given according to Kurihara et al. (13). Special run of sequencing confirmed that subtilisin A-50 from *B. subtilis* A-50 has exactly the same NH₂-terminal sequence as subtilisin BPN'. ISP, Intracellular serine protease of *B. subtilis* A-50 (molecular form Glu-ISP-A).

^b Asp-30 corresponds to Asp-32 in subtilisins, which belongs to the active site of these proteases.

^c Presumably, this residue is deleted in ISP sequence as compared with secretory subtilisins.

chains of these residues of ISP form a cluster of functional groups capable of binding calcium ions shown to be indispensable for ISP. The exact functional role of these peculiar structural features of ISP remains to be defined, but eventually they might be important for interaction with the cell membrane through calcium bridges. The finding of a membrane-bound ISP form confirms this suggestion.

The sequence of the main component of ISP-A, starting with glutamic acid residue (abbreviated as Glu-ISP-A), is two amino acid residues shorter than that of secretory subtilisins. The minor component of ISP-A, starting with asparagine residue (Asn-ISP-A), is three residues longer at its NH₂-terminus than the main component, Glu-ISP-A. Obviously, we face the known phenomenon of protein "ragged" ends. The presence of these two forms has to be explained by a posttranslational modification of NH₂ termini. One explanation might be partial removal, e.g. by aminopeptidase, of NH₂-terminal amino acids after the splitting off the NH₂-terminal methionine residue. An alternative explanation might be the appearance of both forms, Asn-ISP-A and Glu-ISP-A, as a result of the limited proteolysis of an unknown ISP precursor. Such an ambiguous activation pattern is well known for carboxylic proteases, e.g., porcine pepsin (38).

Thus, the following molecular forms of ISP were detected: Glu-ISP-A, Asn-ISP-A, and Glu-ISP-B. The existence of Asn-ISP-B cannot be excluded, because the amount of the material subjected to sequencing might be insufficient for the detection of this minor component.

ISP undoubtedly belongs to the class of serine proteases as shown by its full inactivation by PMSF, a common inhibitor of these enzymes. Ethylenediaminetetraacetic acid and ethylene-glycol-bis(β -aminoethyl ether)-*N,N*-tetraacetic acid also completely inactivate ISP, apparently due to its destabilization caused by the removal of calcium ions. When compared with subtilisin BPN', ISP showed much higher specific activity against chromogenic peptide and ester substrates, hemoglobin and bovine serum albumin, was very low. Perhaps this might be explained by the low pI of ISP (4.3, compared with pI 8.0 to 8.1 for the main form of secretory subtilisins), which leads to an unfavorable shift of enzyme electrostatic charge under the conditions of the activity assay. Nevertheless, ISP seems to be much more specific in its action on the protein substrates when compared with secretory subtilisins. Thus, it practically does not act on the mixture of *B. subtilis* intracellular proteins in vitro, although such a protein mixture is effectively cleaved by subtilisin BPN'

under the same set of conditions (manuscript in preparation).

ISP studied in the present work possesses common traits with the acidic intracellular serine protease found in *B. subtilis* 168 by Reyssset and Millet (30). Unfortunately, the latter enzyme has been scarcely characterized from the chemical point of view, complicating a strict comparison. It seems that the enzyme described by these authors might be identical with ISP.

The exact biological functions of ISP have not been elucidated. In any case it seems to be a more reasonable candidate for the role of protein-catabolizing enzyme and/or modifying agent than secretory subtilisins. A low activity of ISP against protein substrates allows one to consider ISP as a "modifying" enzyme, rather than a "degrading" one. Taking in account the appearance of ISP at the beginning of the sporulation phase, this protease may serve for some specific processing functions. Presumably, ISP may be involved in processing of spore coat proteins like the serine protease recently detected in *B. cereus* cells (5).

All data discussed above show that the *B. subtilis* genome contains at least two homologous structural genes for serine proteases, intracellular serine protease and secretory subtilisin, that arose obviously as the result of ancestor gene duplication. To our knowledge this is the first gene duplication in a bacterial genome confirmed by sequence data. The presence of two homologous genes in the cell might eventually accelerate the evolution of this particular type of enzymes not only by the loosening of selective constraints (24), but also by creation of numerous sequence variants by means of intragenic recombination. It is tempting to assume that the acceleration of this kind might be the reason for the surprisingly high rate of subtilisin evolution.

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LITERATURE CITED

1. Aiyappa, P. S., L. J. Traficante, and J. O. Lampen. 1977. Penicillinase-releasing protease of *Bacillus licheniformis*: purification and general properties. *J. Bacteriol.* 129:191-197.
2. Andrews, P. 1964. Estimation of the molecular weight of proteins by Sephadex gel-filtration. *Biochem. J.* 91:222-233.
3. Bott, K. F. 1976. Regulation of bacterial sporulation, p. 419-436. In K. D. Macdonald (ed.), Second international symposium on the genetics of industrial microorganisms. Academic Press, London.
4. Chaloupka, Y., M. Stranadova, and V. Zalabak. 1977. Intracellular proteolytic activity during sporulation of *Bacillus megaterium*. *Folia Microbiol.* 22:1-11.
5. Cheng, Y.-S. E., and A. I. Aronson. 1977. Alterations of

- spore coat processing and protein turnover in a *Bacillus cereus* mutant with a defective postexponential intracellular protease. *Proc. Natl. Acad. Sci. U.S.A.* **74**:1254-1258.
6. Dancer, B. N., and J. Mandelstam. 1975. Production and possible function of serine protease during sporulation of *Bacillus subtilis*. *J. Bacteriol.* **121**:406-410.
 7. Davis, B. J. 1964. Disc electrophoresis. II. Methods and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**:404-427.
 8. Glenn, A. R. 1976. Production of extracellular proteins by bacteria. *Annu. Rev. Microbiol.* **30**:41-62.
 9. 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.
 10. Hermodson, N. A., L. H. Ericsson, K. Titani, H. Neurath, and K. A. Walsh. 1972. Application of sequenator analysis to the study of proteins. *Biochemistry* **11**:4493-4502.
 11. Hiroishi, S., and H. Kadota. 1976. Intracellular proteinases of *Bacillus subtilis*. *Agr. Biol. Chem.* **40**:1047-1049.
 12. Hirs, C. H. W. 1967. Determination of cystine as cysteic acid. *Methods Enzymol.* **11**:59-62.
 13. Kurihara, M., F. S. Markland, and E. L. Smith. 1972. Subtilisin amylosacchariticus. III. Isolation and sequence of the chymotryptic peptides and the complete amino acid sequence. *J. Biol. Chem.* **247**:5619-5631.
 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. Lyublinskaya, L. A., S. V. Belyaev, A. Y. Strongin, L. F. Matyash, E. D. Levin, and V. M. Stepanov. 1974. A new chromogenic substrate for subtilisin. *Anal. Biochem.* **62**:371-376.
 16. Lyublinskaya, L. A., L. D. Yakusheva, and V. M. Stepanov. 1977. Synthesis of subtilisin peptide substrates and their analogs. *Bioorganicheskaya Khimiya* **3**:273-279.
 17. Markland, F. S., and E. L. Smith. 1971. Subtilisins: primary structure, chemical and physical properties, p. 561-608. *In* P. Boyer (ed.), *Enzymes*, vol. 3. Academic Press, New York.
 18. Mendez, E., and C. Y. Lai. 1975. Regeneration of amino acids from thiazolinones formed in the Edman degradation. *Anal. Biochem.* **68**:47-53.
 19. Millet, J. 1970. Characterization of proteinases excreted by *Bacillus subtilis* Marburg strain during sporulation. *J. Appl. Bacteriol.* **33**:207-219.
 20. Millet, J., P. Kerjan, J.-P. Aubert, and J. Szulmajster. 1972. Proteolytic conversion *in vitro* of *Bacillus subtilis* vegetative RNA polymerase into homologous spore enzyme. *FEBS Lett.* **23**:47-50.
 21. Millet, J., M. Larribe, and J.-P. Aubert. 1976. Mutant thermosensible de *B. subtilis* affecté dans le sporulation et la sérylprotéase extracellulaire. *Biochimie* **58**:109-117.
 22. Munnely, K. P., and A. Kapoor. 1976. Characterization of an alkaline subtilopeptidase type Pfizer. *Int. J. Pept. Protein Res.* **8**:141-153.
 23. Nakamura, K., A. Matsushima, and K. Horikoshi. 1973. The state of amino acid residues in alkaline protease produced by *Bacillus* no. 221. *Agr. Biol. Chem.* **37**:1261-1267.
 24. Ohno, S. 1970. Evolution by gene duplication. Springer-Verlag, New York.
 25. Pacaud, M., L. Sibilli, and G. Le Bras. 1976. Protease I from *Escherichia coli*. Some physico-chemical properties and substrate specificity. *Eur. J. Biochem.* **69**:141-151.
 26. Piggot, P. J., and J. G. Coote. 1976. Genetic aspects of bacterial endospore formation. *Bacteriol. Rev.* **40**:908-962.
 27. Pine, M. J. 1972. Turnover of intracellular proteins. *Annu. Rev. Microbiol.* **26**:103-126.
 28. Pisano, J. J., T. J. Bronzert, and H. B. Brewer. 1972. Advances in gas-chromatographic analysis of amino acid phenyl-*N*-methylthiohydantoins. *Anal. Biochem.* **45**:43-59.
 29. Prestidge, L., V. Gage, and J. Spizizen. 1971. Protease activities during the course of sporulation in *Bacillus subtilis*. *J. Bacteriol.* **107**:815-823.
 30. Reysset, G., and J. Millet. 1972. Characterization of an intracellular protease in *B. subtilis* during sporulation. *Biochem. Biophys. Res. Commun.* **49**:328-334.
 31. Setlow, P. 1976. Purification and properties of a specific proteolytic enzyme present in spores of *Bacillus megaterium*. *J. Biol. Chem.* **251**:7853-7862.
 32. Spizizen, J. 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. *Proc. Natl. Acad. Sci. U.S.A.* **44**:1072-1078.
 33. Stepanov, V. M., L. S. Lobareva, G. N. Rudenskaya, V. P. Borovikova, G. G. Kovaleva, and G. I. Lavrenova. 1977. Gramicidin S as a specific ligand for affinity chromatography of carboxylic proteinases. *Bioorganicheskaya Khimiya* **3**:831-835.
 34. Stepanov, V. M., A. Y. Strongin, L. S. Izotova, 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.
 35. Strongin, A. Y., Z. T. Abramov, E. D. Levin, and V. M. Stepanov. 1975. The multiple forms of the Novo type subtilisin. *Bioorganicheskaya Khimiya* **1**:1441-1448.
 36. Svendsen, I. 1976. Chemical modifications of the subtilisins with special reference to the binding of large substrates. A review. *Carlsberg Res. Commun.* **41**:237-291.
 37. Szulmajster, J., and E. Keryer. 1975. Isolation and properties of thermosensitive sporulation mutants of *Bacillus subtilis* deficient in intracellular protease activity, p. 271-278. *In* P. Gerhardt, R. N. Costilow, and H. L. Sadoff (ed.), *Spores VI*. American Society for Microbiology, Washington, D.C.
 38. Tang, J., P. Sepulveda, J. Marcinszyn, K. C. S. Chen, W.-Y. Huang, N. Tao, D. Liu, and J. P. Lanier. 1973. Amino acid sequence of porcine pepsin. *Proc. Natl. Acad. Sci. U.S.A.* **70**:3437-3439.
 39. Vesterberg, O., L. Hansen, and A. Sjösten. 1977. Staining of proteins after isoelectric focusing in gels by new procedure. *Biochim. Biophys. Acta* **491**:160-167.
 40. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**:4406-4412.