CHARACTERIZATION OF AN INTRACELLULAR PROTEASE IN B. SUBTILIS DURING SPORULATION.

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An intracellular serylprotease has been characterized in B. subtilis, Marburg strain. Like the extracellular serylprotease of that strain, it occurs only during sporulation, but otherwise differs in many characteristics, including its migration on electrophoresis, its absolute requirement for calcium and its narrower specificity toward esters. On the other hand, it is quite similar to the cytoplasmic serylprotease of B. megaterium also synthesized during sporulation. Since the latter enzyme can specifically modify the B. subtilis vegetative RNA polymerase in vitro, it is conceivable that the intracellular B. subtilis serylprotease studied here carries out this modification in vivo.

Most studies of Bacilli proteases have dealt with extracellular enzymes whose role in sporulation is still a matter of dispute (1). Only one protease, a metalloenzyme, is excreted in B. megaterium, yet a mutant completely devoid of extracellular proteolytic activity has the same sporulation pattern as the wild strain (2). In the same species, a cytoplasmic serylprotease, synthesized during sporulation, has been characterized (3) and has been shown to convert B. subtilis vegetative RNA polymerase into the homologous spore enzyme in vitro (4). In B. subtilis, three extracellular proteases are excreted during sporulation: a metalloprotease, a serylprotease and an esterase endowed with a low proteolytic activity (5). It was shown previously that the metalloprotease is not required for sporulation (6). The role of the serylprotease is more controversial. According to Leighton et al. (7),the extracellular serylprotease is responsible for the RNA polymerase modification that occurs during sporulation. According to Millet et al.(4),on the other hand, even very low concentrations of this enzyme almost completely degrade the polymerase in vitro. This makes it unlikely that the extracellular serylprotease is responsible for specific modifications. The physiological role of the third

extracellular enzyme, the esterase, remains an open question.

If one believes that sporulation-specific, protease-catalyzed enzyme modifications follow the same general pattern in all Bacilli species, one would expect B. subtilis to synthesize an intracellular protease similar to that found in B. megaterium . Results presented here show that this is indeed the case and that the intracellular serylprotease synthesized during sporulation in B. subtilis differs from the extracellular serylprotease of this organism but is similar to the intracellular serylprotease of B. megaterium.

MATERIAL AND METHODS. Mutant 512 derived from B. subtilis, Marburg strain, was used in this study.This mutant is deficient in the extracellular metalloprotease, but production of the other extracellular enzymes and sporulation are normal (6). The strain was grown in Difco nutrient broth (8) at 37°C.

Proteolytic activity was determined with azocoll (9) and azocasein (5) as substrates. One unit of activity was equivalent to the hydrolysis of 1 mg azocoll or 1 mg azocasein per minute at 37°C. Esterolytic activity was studied with the following substrates : Z-tyrosine p-nitrophenylester (Z-tyr-ONP), benzoyltyrosine ethylester (BTEE), acetyltyrosine ethylester (ATEE), acetylphenylalanine 2-naphtylester (APNE), tosylarginine methylester (TAME), and benzoylarginine ethylester (BAEE).

Polyacrylamide gel electrophoresis was performed as described by Ornstein (10). A 7 cm resolving gel (7% acrylamide) was used. Electrophoreses were carried out by subjecting the gels to 2.5 mA per gel for 1.5 hr in 5 mM Tris-glycine buffer, pH 8.4 at 4°C.

Protein concentration was determined either spectrophotometrically (11) or by the method of Lowry et al. (12).

RESULTS. Protease purification. Strain 512 was grown in a 15 liter fermentor. Bacteria were harvested at t_2. t_o is defined as the time at which exponential growth ceases; t_1, t_2 etc. are times in hrs after t_o. In order to eliminate contaminating extracellular proteases, bacteria were washed twice: first with a buffer containing

TABLE 1. Purification of the intracellular protease of B. subtilis.

Step	Volume (ml)	Total activity (Azocoll units)	Total protein (mg)	Specific activity (Azocoll units/mg)
1. Crude extract	173	415	3,460	0.12
2. Dialyzed extract	180	365	2,520	0.14
3. Streptomycin sulf. supernatant	185	360	1,340	0.27
4. Ammonium sulfate precipitate	12	210	242	0.94
5. DEAE cellulose eluate	25	110	10	11.0

20 mM Tris-HCl, pH 7.3, 2mM $CaCl_2$, and 1 M NaCl, then with the same buffer but free of NaCl. All steps thereafter were performed at 4°C.

1. Crude extract preparation. 30 g cells (wet weight) were suspended in 200 ml of a buffer containing 200 mM Tris-HCl, pH 7.3, and 2mM $CaCl_2$. Crude extract was prepared by sonication for 30 min with a Branson sonifier Model B-12 followed by centrifugation at 40,000 g for 30 min.

2. Nucleic acid precipitation. The crude extract was dialyzed against a buffer containing 20 mM Tris-HCl, pH 7.3, and 2 mM $CaCl_2$. Nucleic acids were removed by adding 1 mg streptomycin sulfate per mg protein.

3. Ammonium sulfate fractionation. The protein fraction that precipitated between 0.60 and 0.85 ammonium sulfate saturation was dissolved in a buffer containing 50 mM Tris-HCl, pH 7.3, 10 mM $CaCl_2$ and 5 mM dithiothreitol. The solution was dialyzed against the same buffer.

4. DEAE cellulose chromatography. A DEAE cellulose column (I.5 x

26 cm) was equilibrated with the 50 mM Tris buffer described in the preceding paragraph. Stepwise elution was performed by increasing NaCl concentrations in the same buffer. The excluded protein fraction and that eluted by 100 mM NaCl were discarded. Most of the proteolytic activity was eluted by 125 mM NaCl. Because the protease was unstable in buffers containing low ionic concentrations, the active fraction was collected in tubes to which 1 ml 2 M Tris-HCl buffer, pH 7.3 had been added so that the final concentration was 0.6 M. Results are summarized in Table 1.

Electrophoretic studies. An aliquot of the purified protease (about 40 µg protein) was layered on each of two gels and subjected to electrophoresis. One gel was used for protein staining, the other was cut in slices 2 mm thick which were tested for proteolytic activity. Protein staining revealed the presence of nine separate protein bands. Proteolytic activity was found in a single peak that migrated toward the anode and was located at 4 cm from the start for a total run of 6 cm.

Similar electrophoretic studies were done with the pure extracellular serylprotease produced by the same strain, either alone or mixed with the purified intracellular protease. In both cases the extracellular protease, whose pI is around 8.4, remained at the start.

Electrophoresis of the crude extract showed it not to be contaminated with the extracellular serylprotease and that the proteolytic activity was located in a single peak, the mobility of which was the same as that of the purified protease.

Biochemical properties. When azocasein was used as substrate, a small activity was detected. With synthetic substrates, esterolytic activity was observed on Z-tyr-ONP, BTEE and ATEE, but no activity was detected on APNE, TAME and BAEE. Activities on both azocasein and Z-tyr-ONP were found in the same electrophoretic peak as azocoll activity.

Proteolytic and esterolytic activities were maximum at pH values between 7.3 and 9.0. Both types of activity were totally inhibited by 1 mM phenylmethylsulfonyl fluoride and 5 mM ethylene-

diamine tetraacetate. O-phenanthroline did not have any effect. Omission of Ca^{++} at any step of the purification procedure resulted in total and irreversible loss of activity. The partially purified protease was unstable, but its stability could be increased by keeping the enzyme in 600 mM Tris-HCl, pH 7.3, 10 mM $CaCl_2$, 5 mM dithiothreitol and 50 % glycerol.

Kinetic studies. Strain 512 was grown in 2 liter toxin flasks that contained 0.5 l culture medium. Cells were harvested at various times, washed and disrupted as described above for the crude extract. Proteolytic activity on azocoll was determined in the crude extract. In each case, the absence of the extracellular serylprotease was checked by electrophoresis. Results presented in Table 2 show that the proteolytic activity appears after t_o, reaches a maximum around t_3, then declines.

DISCUSSION. The intracellular serylprotease that occurs in B. subtilis during sporulation seems to be a chymotrypsin like enzyme with restricted specificity, since it hydrolyzes ester bonds of tyrosine derivatives but not those of phenylalanine derivatives.

It is interesting to compare the properties of this protease to those of B. subtilis extracellular serylenzymes and of B. megaterium intracellular serylprotease. In B. subtilis grown in nutrient broth, the intracellular and extracellular proteases appear only during sporulation. Nevertheless the intracellular serylprotease differs from the extracellular serylprotease in several ways: a) it has a lower pI; b) its specificity toward esters is narrower. Some, such as TAME and BAEE, are hydrolyzed by the extracellular serylprotease (5), but not by the intracellular serylprotease; c) its requirement for calcium is absolute. The intracellular protease differs also from the B. subtilis extracellular esterase, whose activity on BTEE is much higher than that of the protease. In contrast to the protease, the esterase has activity on TAME and is not inhibited by EDTA (5).

A comparison of the B. subtilis intracellular protease with the B. megaterium intracellular protease shows that both enzymes

TABLE 2. Kinetics of the intracellular protease of B. subtilis during growth and sporulation.

Time	Specific activity (Azocoll units/mg protein)
Growth: t_{-1}	0
Sporulation:	
$t_{0.15}$	0
t_2	0.35
$t_{3.5}$	0.73
t_5	0.47

have many properties in common. Both are serylenzymes with a calcium requirement, both have the same specificity on synthetic substrates, both have similar electrophoretic mobility. The only marked difference between the two enzymes lies in their activity on azocasein. While the B. megaterium intracellular protease has none, that of B. subtilis, while small, is nevertheless significant. Because the B. megaterium intracellular protease has been shown (4) to modify the B. subtilis vegetative RNA polymerase specifically in vitro, the protease studied here may carry out this modification both in vitro and in vivo. This question is presently under investigation.

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