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Intracellular Proteases of *Bacillus stearothermophilus*

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Cell-free extracts of *Bacillus stearothermophilus* have been shown to exhibit proteolytic activity toward casein as well as specific activity to catalyze the hydrolysis of furylacryloylglycyl-L-leucine amide, furylacryloylglycine, and carbobenzoxylglycine-*p*-nitrophenyl ester, indicating the presence of a neutral proteinase, a carboxypeptidase-like enzyme, and an alkaline proteinase. The neutral proteinase and carboxypeptidase-like activities were separated by gel filtration over Bio-Gel P-60, and both were reversibly inhibited by 1,10-phenanthroline. The esterase activity was inhibited by diisopropylfluorophosphate, which did not affect other enzymatic activities and was insensitive to 1,10-phenanthroline and ethylenediaminetetraacetic acid.

Numerous microorganisms have been shown to produce extracellular proteolytic enzymes (6). The bacilli produce extracellular proteinases of various kinds, including the alkaline proteinases which are serine hydrolases and the metallo neutral proteinases (8-12). Few studies, however, have been reported concerning the intracellular nature of these enzymes (2, 7, 10). *Bacillus stearothermophilus* produces a thermostable, extracellular metallo neutral proteinase. Extracts of a strain of this organism contain neutral proteinase activity, a carboxypeptidase-like enzyme, and an esterase, perhaps an alkaline proteinase. These studies are reported here.

MATERIALS AND METHODS

Microorganism. *B. stearothermophilus* NRRL B-3880 was used in this study. Stock cultures were maintained on agar slants of starch (3%), soy flour (1.5%), yeast extract (0.2%), salts (0.5%) (*p*H 7.1), incubated at 55 C for 24 hr, and frozen.

Inoculum. The inoculum was prepared from the growth of a slant suspended in 5 ml of sterile nutrient broth. Three 500-ml portions of nutrient broth in 2-liter Erlenmeyer flasks were inoculated with 1 ml of the suspension and placed in a shaker incubator at 55 C for 12 hr. Six 2-liter baffled shaker flasks containing 500 ml of the same medium were inoculated with 30 ml of nutrient broth growth. They were placed in a shaker incubator at 55 C, rotating at 150 rev/min, and purged with 500 ml of air per minute. After 8 hr, the content of the shaker flasks was used to inoculate the fermentor.

Fermentor. The 150-liter fermentor was a stainless-steel jacketed vessel, agitated (270 rev/min) with one impeller above the air sparger and aerated with 40 liters/ min (standard conditions) at 1.5 atm gauge and at 57 C. The growth medium consisted of the following: starch (Corn Products, Globe 3005), 36 g/liter; soy flour (Central Soya, Soyafluff W), 18 g/liter; yeast extract (Difco), 2 g/liter; KH_2PO_4, 0.5 g/liter; K_2HPO_4, 3.5 g/liter; $MgSO_4$, 0.1 g/liter; $CaCl_2$, 0.2 g/liter; antifoam (Mazer, Mazu DFO), 3 g/liter (*p*H 7.0).

Preparation of cell extracts. After 21 hr of growth, cells were harvested by centrifugation and repeatedly washed with 0.1 M KCl until the washes were free of color and enzyme activity. To 150 g of cells suspended in 150 ml of a *p*H 7.0 HEPES-HCl buffer (0.1 M), 1 ml of lysozyme solution (11 mg) was added. After incubation for 1 hr, another 100-ml portion of the same buffer was added, and the mixture was allowed to stand for another 2 hr. Portions of the digested suspension were mixed with an equal weight of glass powder and blended in a chilled Waring blendor for about 3 to 4 min. Centrifugation for 30 min at 34,800 $\times$ *g* yielded 300 ml of cell-free extract.

Enzyme assays. Proteinase activity was determined by modification (5) of the Anson method (1) with casein as substrate. Neutral proteinase activity was determined by using furylacryloylglycyl-L-leucine amide (FA-gly-leu-NH_2; 9.76×10^{-4} M) in *p*H 7.2 HEPES buffer (0.1 M). The reaction was monitored spectrophotometrically at 345 nm. Synthesis of substrate and details of methods have been described (3, 4). The hydrolysis of furylacryloylglycine (FA-gly) and furylacryloylglycyl-L-leucine (FA-gly-leu) was monitored by the same method. Amino acid analyses were performed in an amino acid analyzer (Beckman model 120C).

Bio-Gel P-60 gel filtration. Bio-Gel P-60 (100 to 200 mesh) was purchased from Bio-Rad Laboratories, Richmond, Calif. The beads were equilibrated with 0.1 M KCl containing 10^{-2} M $CaCl_2$ (*p*H not adjusted) and packed into a column 2.5 by 34 cm. Elution was carried out with the equilibrating solution.

RESULTS AND DISCUSSION

Cell-free extract exhibited proteinase activity with casein as substrate and catalyzed the hy-

TABLE 1. *Distribution of enzymatic activity towards FA-gly-leu-NH_2 and FA-gly in isopropyl alcohol fractions of intracellular extracts of Bacillus stearothermophilus*

Fraction	Initial rate (ΔA_{345}/min)	
	FA-gly-leu-NH_2[a]	FA-gly[b]
I	13.3×10^{-4}	6.2×10^{-3}
II	5.7×10^{-4}	25.4×10^{-3}

[a] Concentration: 9.76×10^{-4} M FA-gly-leu-NH_2 in *p*H 7.2 HEPES buffer (0.1 M).

[b] Concentration: 3.47×10^{-3} M FA-gly in *p*H 7.2 HEPES buffer (0.1 M).

drolysis of FA-gly-leu-NH_2, indicating the presence of a neutral proteinase. To check that the gly-leu bond had been cleaved, an analysis of the products was made after reaction by submitting the solution to amino acid determination. If only the gly-leu bond had been cleaved, no free glycine or free leucine would have been detected. The analysis indicated the presence of an equivalent amount of glycine to the starting FA-gly-leu-NH_2. This indicated that both the gly-leu and FA-gly bonds were cleaved. Since the neutral proteinase cleaved only the gly-leu bond, the presence of an additional enzyme which would cleave the FA-gly bond, perhaps a carboxypeptidase-like enzyme, was suggested. To check this, the cell-free extract was reacted with FA-gly and FA-gly-leu. Amino acid analyses yielded equal amounts of glycine and leucine from FA-gly-leu and a stoichiometric amount of glycine from FA-gly. Since the neutral proteinase could not catalyze the hydrolysis of FA-gly and only very slowly hydrolyze FA-gly-leu, the results suggested that both a neutral proteinase-like enzyme and a carboxypeptidase-like enzyme were present in the extracts.

To determine if these two activities could be separated, a simple isopropyl alcohol fractionation of cell-free extract was carried out. Fifty milliliters of extract was precipitated with one volume of isopropyl alcohol at 0 C. A second fraction obtained by the addition of a second volume of isopropyl alcohol was also obtained. Table 1 shows the relative activity towards FA-gly-leu-NH_2 and FA-gly for these two fractions. Although fraction 1 contained over twofold the activity of fraction 2 towards FA-gly-leu-NH_2 substrate, the activity towards FA-gly was four fold as concentrated in fraction 2 as fraction 1. This would indicate that the two activities could be separated.

Fraction 2 also exhibited esterase activity towards carbobenzoxylglycine-*p*-nitrophenyl ester (Z-glyONP) in phosphate buffer (0.1 M, *p*H 6.0). The esterase activity was totally inhibited by treatment with diisopropylfluorophosphate (DFP), but the activity towards FA-gly-leu-NH_2, FA-gly, and FA-gly-leu was not affected. The DFP-treated enzyme was tested for caseinase activity. After 2 hr of incubation with DFP at 10^{-2} M, 86 to 93% of the casein proteinase activity still remained although all of the esterase activity was destroyed. The esterase activity was not inhibited by 2×10^{-3} M 1,10-phenanthroline nor 3×10^{-3} M ethylenediaminetetraacetic acid. An attempt was made to separate the neutral proteinase activity from the FA-gly hydrolyzing activity by use of gel filtration over Bio-Gel P-60. Seventy-one milligrams of lyophilized fraction 2 in 3 ml of 0.1 M KCl (0.01 M $CaCl_2$) was placed on a column (2.5 by 34 cm) and washed with the same. Two-milliliter fractions were collected and assayed with GA-gly-leu-NH_2 and Fa-gly.

Figure 1 shows the elution profile of this material over Bio-Gel P-60. The FA-gly hydrolyzing activity and the FA-gly-leu-NH_2 hydrolyzing activity were completely separated by this technique. Tube numbers 9 to 18 were pooled and lyophilized to yield fraction A and tube numbers 35 to 54 were pooled and lyophilized to yield fraction B. Fraction A which contained FA-gly hydrolyzing activity had negligible FA-gly-leu-NH_2 activity, whereas fraction B contained the FA-gly-leu-NH_2 activity without any detectable FA-gly activity.

The K_m for the enzyme-catalyzed hydrolysis of FA-gly was determined from single complete reactions to be $5.25 \pm 0.10 \times 10^{-5}$ M. The substrate concentration was 2.209×10^{-3} M FA-gly in *p*H 7.2 HEPES buffer (0.1 M). A Fortran IV program was used to obtain complete Lineweaver-

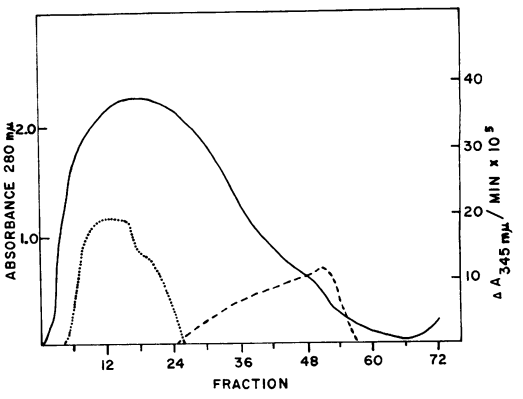

FIG. 1. *Gel filtration of fraction 2 over Bio-Gel P-60. All fractions were assayed with FA-gly-leu-NH_2 (– – –) and FA-gly (· · ·). Absorbance, 280 nm* (——).

Burk plots from single reactions run to completion (4).

Incubation of fraction A with 2×10^{-3} M 1,10-phenanthroline for 30 min resulted in 100% inhibition of the FA-gly activity. The substrate also contained this concentration of chelator. Addition of cobalt ions to a final concentration of 0.012 M reactivated the enzyme to 50% of control. Analysis of products indicated a stoichiometric amount of glycine to the starting FA-gly. Incubation of fraction A with FA-gly-leu yielded equal molar amounts of glycine and leucine by amino acid analysis (6.2 μmoles of glycine; 6.8 μmoles of leucine).

Fraction B was also completely inhibited with respect to its FA-gly-leu-NH_2 hydrolyzing activity in the presence of 10^{-3} to 2×10^{-3} M 1,10-phenanthroline. Addition of cobalt ions to a final concentration of 1.57×10^{-2} M yielded 87% reactivation of the control. This would indicate that fraction B represented an intracellular neutral proteinase capable of cleaving only a neutral proteinase substrate such as FA-gly-leu-NH_2 and was reversibly sensitive to a chelator (1,10-phenanthroline) which is a property shared with other extracellular neutral proteinase. Fraction A, however, represented a carboxypeptidase-like enzyme that was reversibly inhibited by 1,10-phenanthroline. It did not cleave a dipeptide in which the carboxyl was blocked in an amide, but catalyzed the hydrolysis of dipeptides or single amino acids bound to furylacrylic acid if a free carboxyl group was present. Both FA-gly and FA-gly-leu were substrates for this enzyme. Carboxypeptidase A also catalyzed the hydrolysis of FA-gly-leu.

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