Regulation of the Formation of Proteinases in

*Bacillus megaterium*

V. Characterization of Two Megaterioproteinases Differing in the Control of their Synthesis

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**ABSTRACT.** The exocellular proteinases from the asporogenic and sporogenic strain of *Bacillus megaterium* KM were purified and characterized. They are both neutral metalloenzymes, having an optimum pH of 7.2. The bivalent metal cations, particularly calcium or magnesium, are essential for their activity. The curve of the relationship between the reaction velocity and the concentrations of Ca$^{2+}$ resembles the Michaelis curve for substrate concentration. The enzymes also require metal cations for their stability. Both proteinases are inactivated by o-phenanthroline (1 mM) and sodium EDTA (2 mM) and are resistant against diisopropyl fluorophosphate (1 mM) and sodium-p-chloromercuribenzoate (1 mM) treatment. In spite of the difference in biochemical regulation of their synthesis, these exocellular proteinases seem to be similar. The terms, megaterioproteinase A and megaterioproteinase S have been proposed for these enzymes.

The synthesis of exocellular proteinase of asporogenic *Bacillus megaterium* KM is probably controlled by repression by amino acids or their metabolites, together with glucose, and the same is valid also for the strain MA (Chaloupka et al., 1963; Millet and Aubert, 1969; Chaloupka, 1969; Fayyaz Ud Din and Chaloupka, 1970). In the sporogenic strain, amino acids stimulate the formation of its proteinase in the absence of glucose (Fayyaz Ud Din and Chaloupka, 1969). Because of the apparent difference in the biochemical regulation of the synthesis of these two proteinases, the enzyme characteristics of both were studied. The present investigation describes the purification procedure, the enzyme kinetics and enzymatic properties of these proteinases.

**MATERIALS AND METHODS**

*Organism.* The asporogenic strain of *Bacillus megaterium* KM was supplied by Chester Beatty Res. Inst. London and the sporogenic strain by Prof. A. Aronson, Purdue Univ., Lafayette, Ind. USA.

*Enzyme biosynthesis.* The proteinases were synthesized in a complex medium composed of yeast, glucose and CaCl$_2$ with aeration at 30°C. The culture was grown for 30 and 45 h in the case of asporogenic and sporogenic strain respectively. This produced a satisfactory harvest of enzymes, particularly of the proteinase of the asporogenic strain. In many experiments, an amount of enzymes corresponding to over 10 tyrosine units in 1 ml of cell-free culture liquid of asporogenous strain was obtained.
Buffers. Buffer A: Tris-HCl 0.1M, pH 7.2.  
Buffer B: Tris-HCl 0.1M, pH 7.2 containing 2mM of CaCl₂.  
Buffer C: Tris-maleate 0.05M.

Enzyme assays. Proteolytic activity was determined mainly with casein as substrate on the basis of the Kunitz method (1947). The standard reaction mixture contained 1.9 ml of buffer A, 0.1 ml of CaCl₂ (6 × 10⁻⁵M) in buffer A, 5 µl of purified enzyme preparation and 1 ml of 1% casein (in buffer A). The reaction was run at 37°C for 10 min. The blanks and controls were run simultaneously. Its activity was expressed in tyrosine units (Millet, 1969).

The proteolytic activity was also determined at pH 7.2 with haemoglobin as substrate. Urea-denatured haemoglobin was used and the assay was done according to the Anson method (1938).

Esterase assay was based on the method of Martin et al. (1959) and Applebaum et al. (1964). 0.1 ml of 2mM carbobenzoxy L-tyrosine-p-nitrophenol ester was added to the mixture containing 1.8 ml of buffer B and 0.1 ml of enzyme solution (0.05 units). After incubation for 10 min at 37°C the reaction was stopped by adding 2 ml of 10% TCA. The extinction was measured at 320 nm.

Protein determination. The method of Lowry et al. (1951) was used with casein (Hammarsten) as standard.

Fractionation of the enzyme preparations

(a) Supernatant of culture medium of the asporogenic strain. The supernatant was cooled to −2°C and precipitated by the addition of 2 volumes of alcohol 96% previously cooled to −25°C. After 3−5 h at −25°C the clear supernatant was carefully removed by decanting. The remainder containing the precipitate was centrifuged at 20,000 g for 20 min at −20°C. The precipitate was immediately suspended in a suitable amount of ice cold buffer B and then dialyzed while stirring constantly for 30 min against the same buffer. The suspension was then centrifuged to eliminate the remaining precipitate. The supernatant containing enzyme was fractionated further with solid ammonium sulphate added while stirring slowly until a concentration 50% (w/V).

The mixture was kept at 0°C overnight and the precipitate formed was collected by centrifugation. The pellet was redissolved in cold buffer B and dialyzed against the same buffer for 30 min at 0°C while stirring.

(b) Supernatant of culture medium of the sporogenic strain: The supernatant was first cooled to 0°C and then fractionated by the addition of ammonium sulphate until 50% (w/V) at +2°C; the mixture was finally treated as described above (a).

Concentration of enzyme solution by sucrose. The small volumes (20−30 ml) of enzyme preparations were concentrated satisfactorily by concentration dialysis, using sucrose as water-absorbing material. The concentrated enzyme solution was then dialyzed in cold buffer B to eliminate the sucrose. The whole process was conducted in the cold room.

Gel filtration of enzyme preparations. Sephadex G-100 was allowed to swell for 3 days in excess water and then packed into columns 1.5 x 30 cm. The columns were equilibrated overnight with buffer B and then the enzyme solution (7 ml) was applied. The elution was conducted at 2°C by the above-mentioned buffer at a flow rate of 10 ml/h. The effluent was collected in 3 ml samples and recorded at 280 nm.

Sephadex G-50 coarse was allowed to swell in an excess of water for 3 h at room temperature. The remainder of the process of handling was the same as described