ENZYMOLOGICAL DIFFERENCES OF α-AMYLASE FROM BACILLUS STEAROTHERMOPHILUS GROWN AT 37° C AND 55° C*

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Summary: α-Amylase purified from the culture filtrate of Bacillus stearothermophilus grown at 55° C showed remarkably higher heat stability and had smaller Km values for starch than that from the same bacterium grown at 37° C. These differences may reflect the structural alteration of the enzyme protein depending upon culture temperature.

An α-amylase purified from the culture filtrate of B. stearothermophilus showed remarkably higher heat stability and higher optimal temperature when compared with that from B. subtilis (1). On the other hand, an aldolase purified from fishes living in the antarctic sea was more heat labile than those from ordinary animals (2). These and other data arouse our interest to know whether enzymes from an organism grown at different temperatures may have alterations in heat stability or in other characteristics.

A derivative of B. stearothermophilus, strain ATCC 12980, was isolated by the author which grows considerably well at 37° C (3). α-Amylase was purified from the culture filtrate of this strain grown either at 37° C or at 55° C. Enzymological characters of the two preparations were comparatively investigated and some of the preliminary results are reported here.

Materials and Methods

B. stearothermophilus, BS-1, a facultative derivative of ATCC 12980, was

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grown overnight at 55° C in a semi-defined medium, Med-121 (4), in a cotton-stoppered culture flask with reciprocal shaking at a speed of 135 cycles per min. 1 ml of this overnight culture was inoculated into 200 ml each of Med-121 prepared in cotton-stoppered 500 ml culture flasks and shaken at either 55° C for 14 hours or 37° C for 38 hours. The culture was centrifuged at 15,000 rpm in a continuous flow rotor and the clear supernatant was pooled. To 1 litre of the crude liquor was added 15 g of cellulose powder (Toyo-roshi, grade B) and the pH was brought to 6.5 with 5 N NaOH. 445 g of well ground solid ammonium sulfate were slowly added at 0° C to 1 litre of the crude liquor with gentle stirring and constant adjusting the pH to 6.5. The cellulose precipitate was collected on two sheets of filter paper and α-amylase was eluted by stirring the precipitate in 0.01 M potassium phosphate buffer, pH 6.2, containing 2.5 mM NaCl. The resulting transparent effuluent was then slowly passed through a thin starch film prepared on double sheets of filter paper on a Buchner funnel at a speed of 1 litre per hour, and then the adsorbed enzyme was eluted twice from the starch by incubation of the starch slurry at 60° C for 7 min followed by immediate centrifugation at 10,000 rpm for 20 min. The supernatant was combined, brought to 0° C and dialyzed against 0.005 M calcium acetate for 4 days with several changes of the dialyzing solution. The content of the bag was pipetted out and centrifuged at 25,000 rpm for 30 min in a Spinco L2 ultracentrifuge. The clear, almost colorless supernatant was lyophilized, re-dissolved in ca. 10 ml of doubly distilled water and brought to pH 8.5 with 1 M tris(hydroxymethyl)aminomethane. It was then applied on a DEAE-Sephadex A50 column equilibriated with 0.05 M Tris-HCl, pH 8.5, and eluted with the same buffer. Fractions showing high amylase activity were pooled, dialyzed against several changes of distilled water for 3 days and lyophilized. The purification indices at the last step were 7,200 for 55° C enzyme and 6,900 for 37° C enzyme.

Results and Discussion

Disc-electrophoresis: The two enzyme preparations were analysed by disc-

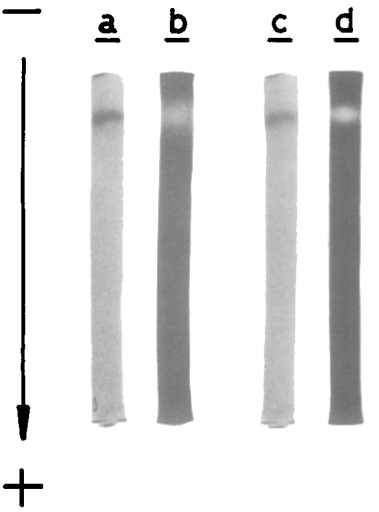

Fig.1: Disc-electrophoresis of 37° C and 55° C enzymes. Protein concentration of the purified enzymes was determined according to Lowry et al. (6) and 100 μg of each enzyme were apllied to the top of gels prepared in glass tubes (5 x 70 mm). Gels were polymerized as described by Gordon (7). Separating gels were 7.5 % gels (pH 8.9) and condensing gels were 2.5 % gels (pH 6.7). Electrophoresis was carried out in the cold (ca. 4° C) at an electric current of 2 mA per gel for 120 min. Gels were either stained with 1 % amino black dissolved in 7 % acetic acid (a and c) or immersed in 2 % soluble starch and kept at 0° C for 4 hr and then incubated and stained as described in text (b and d). a and b for 37° C enzyme, and c and d for 55° C enzyme.

electrophoresis at pH 8.3 on 7.5 % acrylamide gels. Gels were either stained with amino black 10B or immersed in 2 % soluble starch at 0° C for 4 hours and subsequently incubated at 37° C for up to 3 min in 0.05 M potassium phosphate buffer, pH 6.2, containing 12.5 mM NaCl and 0.4 mM $CaCl_2$. The latter gels were then stained with a mixture of 0.15 % iodine and 1.5 % potassium iodide. Results are shown in Fig.1. Both stainings proved the high purity of the two enzyme preparations and the protein bands (a and c) coincided well with the activity bands (b and d). The 37° C and 55° C enzymes are identical with respect to electrophoretic mobility under the conditions employed here.

Temperature and enzyme activity: Both preparations were assayed for their amylase activity at different temperatures according to the assay system

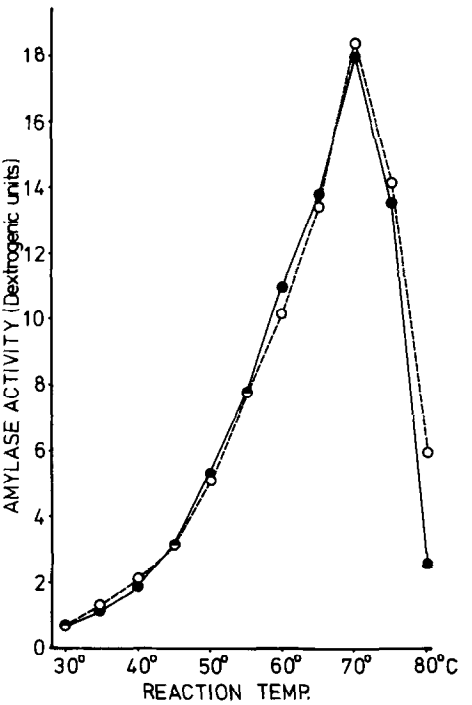

Fig.2: Activity-temperature relationship. Amylase activity was assayed according to Pfueller and Elliott (5) and expressed as dextrogenic units per ml in the ordinate. The abscissa expresses reaction temperature. ●——● 37° C enzyme; O----O 55° C enzyme.

described by Pfueller and Elliott (5). Reaction was continued for 10 min and stopped by the addition of cold 1 N HCl. Results illustrated in Fig.2 show almost identical temperature-activity relationship for the 37° C and 55° C enzymes except that the activity of the former decreases slightly but significantly faster than that of the latter above 70° C. The optimal temperature was 70° C for both enzymes, which was in good agreement with that reported earlier (1). Heat stability of the two preparations was, however, distinctly different from each other as shown in Fig.3. The loss of activity of the 37° C enzyme was 98 %, while that of the 55° C enzyme was 83 % after a 30 min incubation at 80° C in the presence of 5 mM $CaCl_2$ in 0.05 M Tris-HCl buffer, pH 7.4. The kinetics of inactivation was roughly of first order for both enzymes. The presence of $CaCl_2$ was inevitably necessary for stabilization and both enzymes lost almost 100 % of

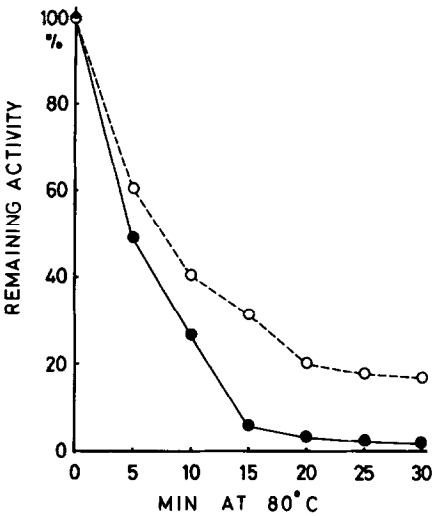

Fig.3: Heat stability of 37° C and 55° C enzymes. Each enzyme was adjusted to 100 μg per ml and incubated at 80° C. At the time indicated, aliquots were removed and chilled to 0° C, amylase activies of them were assayed and expressed as percentage of the untreated control. ●——● 37° C enzyme; O----O 55° C enzyme.

their activity in its absence after 15 min at 80° C.

Km values for starch: Km Values of both enzymes for starch were estimated at 65° C and 37° C. They were 392.0 and 44.8 mg starch per litre for 37° C and 55° C enzymes, respectively, when assayed at 65° C, and 48.5 and 28.3, respectively, when assayed at 37° C as shown in Fig.4. Reproducibility of these values was good in three independent assays. This may reflect the intrinsic difference between these two enzymes.

Four mutants lacking amylase activity have been isolated (4). All of them showed negligible amylase activity when grown at either 55° C or 37° C. Moreover, transformants obtained from them showed amylase activity at almost identical level to the wild type at both temperatures. These facts indicate that the gene specifying the amylase may be one. It is interesting to investigate whether the primary structure of these preparations is different or not. A preliminary result showed differences in the amino acid composition between the two enzymes.

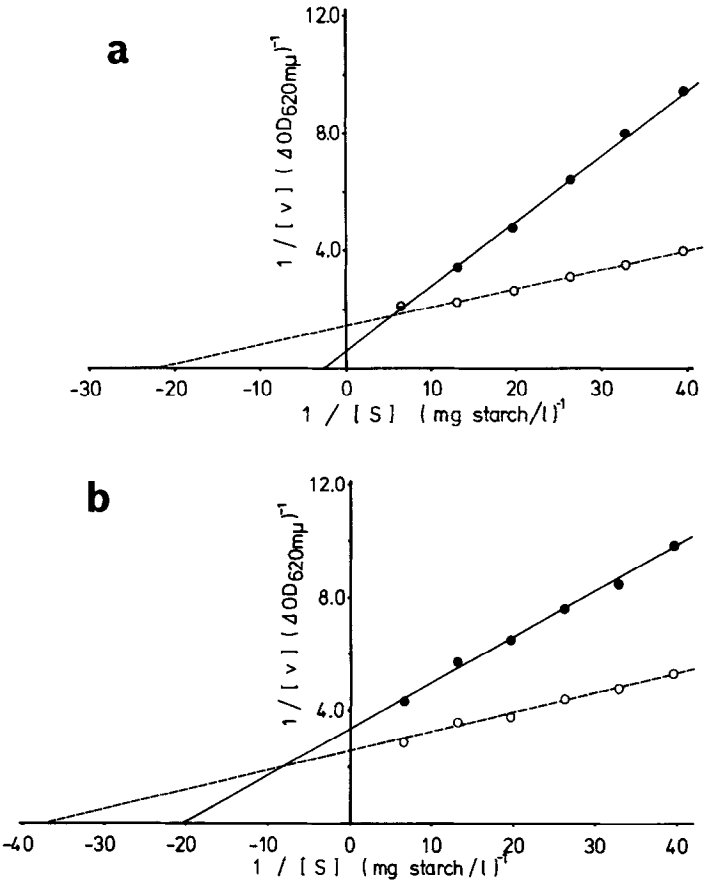

Fig.4: Lineweaver-Burk plottings of 37° C and 55° C enzymes. Amylase activity was assayed either at 65° C (a), or at 37° C (b). Reciprocals of reaction velocity ($OD_{620m\mu}$ in 10 min) were plotted against reciprocals of starch concentration. ●——● 37° C enzyme; O----O 55° C enzyme.

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