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Properties of the Amylase from *Halobacterium halobium*[1]

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Halobacterium halobium amylase had optimal activity at *p*H 6.4 to 6.6 in sodium β-glycerophosphate buffer containing 0.05% NaCl at 55 C; Ca^{2+} was not required. End products from amylose were maltose, maltotriose, and glucose. The amylase, which was devoid of transglucosylase activity, had a multichain attack mechanism.

In searching for amylases that possess unique new properties, we examined enzyme production by 10 halophiles. Strain 3-3, kindly supplied by N. E. Gibbons, Division of Biosciences, National Research Council, Ottawa, Canada, produced more amylase than the other halophiles in media containing 10 to 30% NaCl. We identified strain 3-3 as *Halobacterium halobium;* the amylase produced by this bacterium was partially characterized.

Gibbons (2) basal medium devoid of L-glutamic acid was modified by the addition of 10 μg of $FeSO_4 \cdot 7H_2O$ per ml (10), 0.2% soluble starch, and 0.3% agar; the *p*H was adjusted to 6.5 before sterilization. Since amylase yields were much greater on soft agar than in tubes or shake flasks of broth, sterile medium was poured into petri plates and inoculated on the surface. After incubation in a plastic container at 37 C for 5 days, the amylase was harvested by mixing the soft agar with an equal volume of 10% NaCl in 0.01 M sodium β-glycerophosphate buffer (*p*H 7.0). Agar and cells were removed by centrifugation, and the supernatant was sterilized by filtration through a Seitz filter. The supernatant was concentrated to 0.1 volume in an ultrafilter and washed with glycerophosphate-sodium chloride buffer.

Amylase activity was measured by an iodine-staining (blue-value) method (9). Tests for transglucosylase activity, with 0.5% glucose as a potential glucosyl acceptor, were negative.

The effects of high levels of NaCl on the amylase are shown in Fig. 1. This, and additional data (not shown), indicate that the *H. halobium* amylase resembled the enzymes from moderate halophiles (4, 6) in its ionic requirement of 0.05 to 1.0% NaCl for optimal activity. However, the *H. halobium* amylase was more tolerant than most of the enzymes from moderate halophiles to high NaCl levels, maintaining 33% of maximum activity at a NaCl concentration of 4.0 M.

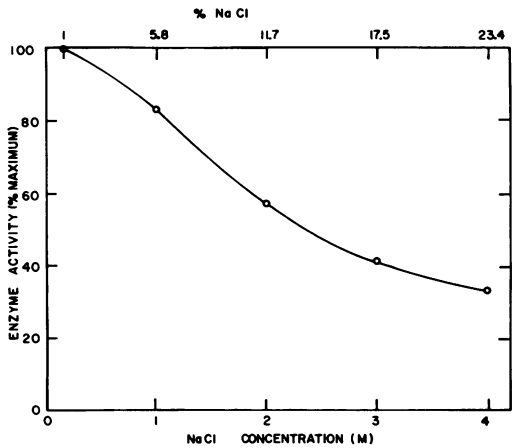

FIG. 1. *Effects of NaCl on the activity of H. halobium amylase. The readings shown were taken after 60 min of incubation at 55 C in 0.01 M sodium β-glycerophosphate buffer containing 0.01 M calcium acetate (pH 6.5).*

When NaCl was removed by extensive dialysis against distilled water, amylase activity was completely lost (Fig. 2). Over 90% of the activity was restored rapidly upon the addition of either 0.25% NaCl or KCl; NaH_2PO_4 did not reactivate the enzyme. Reactivation of *H. halobium* amylase was unlike reactivation of the halophilic malic dehydrogenase studied by Holmes and Halvorson (3) because the amylase of *H. halobium* did not require dialysis against NaCl for reactivation and the malic dehydrogenase required much more salt for activity. A requirement for Ca^{2+} could not be demonstrated, although the Ca^{2+} might

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have been too tightly bound to be removed by dialysis, or sufficient Ca^{2+} for reactivation might have been a contaminant of the reagents used in the reactivation experiments.

Bacillus subtilis amylase was inhibited to a greater extent than *H. halobium* amylase by NaCl (Fig. 2). *B. subtilis* amylase activity was slightly reduced by the presence of 1% NaCl; at a NaCl level of 10%, there was very substantial reduction in *B. subtilis* amylase activity.

The effects of *p*H on the amylase of *H. halobium* at two NaCl concentrations are shown in Fig. 3.

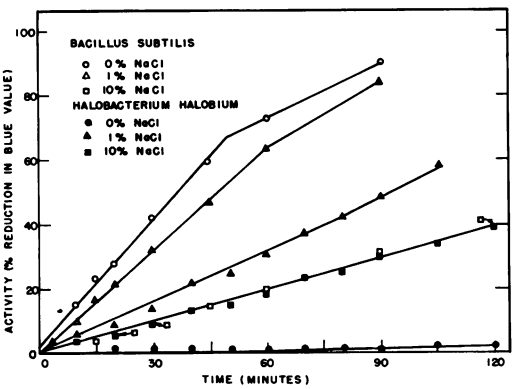

FIG. 2. *Comparison of the effects of 0, 1, and 10% NaCl on the amylases of Bacillus subtilis (type II A crystallized and lyophilized, Sigma Chemical Co.) and H. halobium at 37 C (pH 7.0). B. subtilis amylase was assayed in 0.02 M calcium acetate buffer, and the amylase of H. halobium was assayed in 0.01 M sodium β-glycerophosphate buffer.*

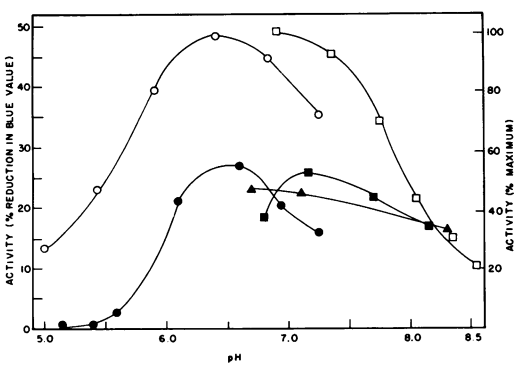

FIG. 3. *Effects of pH on the activity of H. halobium amylase. The assay was made in three different buffers at two salt concentrations. Open symbols, 1% NaCl; closed symbols, 10% NaCl. ○ and ●, Sodium β-glycerophosphate buffer; □ and ■, sodium barbital buffer; ▲, tris(hydroxymethyl)aminomethane buffer. The readings shown were taken after 60 min of incubation at 55 C.*

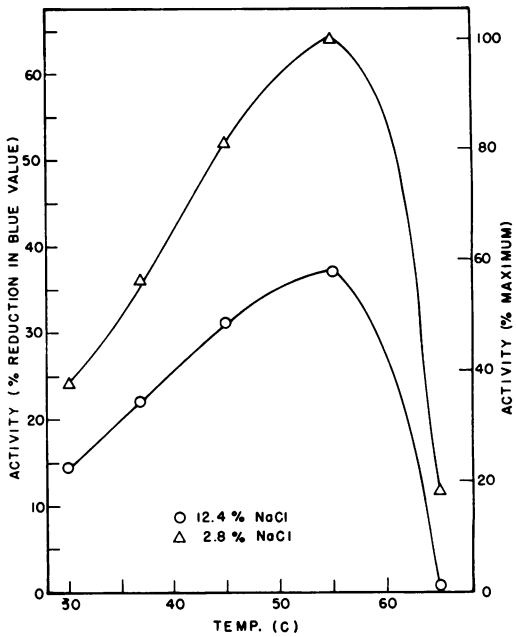

FIG. 4. *Effects of temperature on the activity of H. halobium amylase at two salt concentrations. These readings were taken after 3 hr of incubation in 0.01 M phosphate buffer (pH 7.0).*

The *p*H optimum was 6.4 to 6.6 in β-glycerophosphate buffer. The *p*H optimum seemed to increase when sodium barbital buffer was used.

NaCl had an increasingly inhibitory effect as the temperature of incubation increased (Fig. 4). The optimal temperature, however, was independent of NaCl concentration.

High-temperature paper chromatography (1) was used to determine the type of low-molecular-weight oligosaccharides produced by the amylase of *H. halobium* (Fig. 5). End products were maltose, maltotriose, and glucose. By plotting reductions in blue value against increases in reducing value (9), the tendency for multiple attack (hydrolysis of more than one bond upon an encounter of enzyme with substrate) can be estimated. A blue value/reducing value plot of *H. halobium* amylase action showed that the initial stages of the blue value/reducing value plot were identical to those of an acid-catalyzed reaction, thus indicating that the action of the enzyme was most probably multichain (7; J. F. Robyt, *personal communication*).

The properties exhibited by *H. halobium* amylase differ in two respects from those described by Nachum and Bartholomew (Bacteriol. Proc., p. 137, 1969) for the amylase of another *Halobacterium* sp. Unlike the amylase of *H. halobium*,

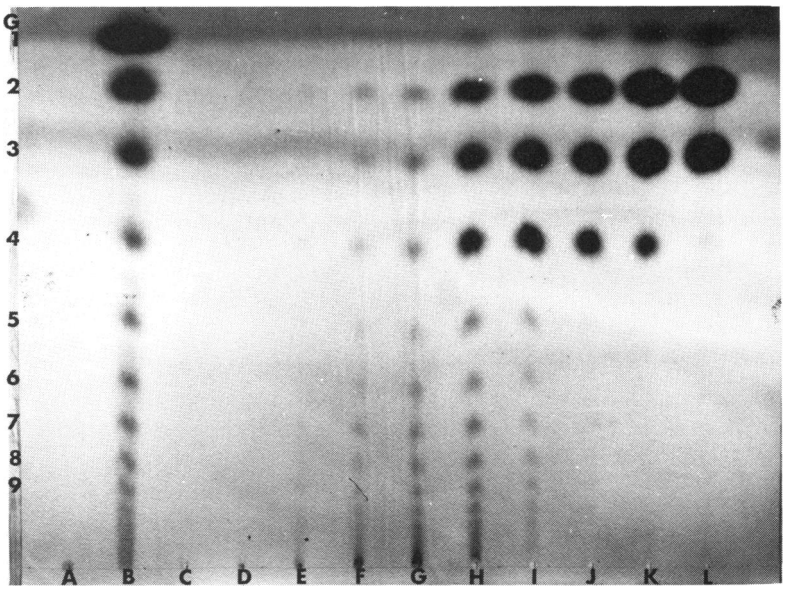

FIG. 5. *High-temperature paper chromatogram (1) of the action pattern of H. halobium amylase. Samples contained 0.5% NaCl, which was removed with 0.5 g of Amberlite MB-3 (Mallinckrodt) per ml for 30 min; 30 μliters of each sample was spotted on Whatman no. 3 paper. Three 8-hr ascents at 70 C were made by using butanol-pyridine-water (3:2:2). A, Boiled enzyme-substrate blank; B, standard G_1–G_{15} (an extensive hydrolysate of starch using Aspergillus oryzae enzymes); C, D, E, F, G, H, I, J, K, and L, 0, 0.25, 0.50, 1, 2, 7, 14, 25, 50, and 104 hr, respectively, reaction time on 0.1% amylose (8). The silver-nitrate dip procedure of Robyt and French (7) was used to develop the chromatogram.*

increasing the NaCl concentration from 5 to 25% resulted in substantial increases in the temperature required for maximal activity, and the temperature needed to affect enzyme inactivation. Nachum and Bartholomew's amylase, like most enzymes from extreme halophiles, required high NaCl concentrations for optimal activity. However, a few enzymes from halophiles, such as the catalase described by Lanyi (5) and the *H. halobium* amylase described here, show maximal activity in less than 1 M NaCl.

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