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ISOLATION AND CHARACTERIZATION OF THERMOSTABLE ALDOLASE FROM A FACULTATIVE THERMOPHILIC BACTERIUM

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Abstract

The aldolase of a facultative thermophilic bacterium, *Bacillus sp.* was characterized. The stability of the enzyme was compared with those of E. coli aldolase and rabbit muscle aldolase. The thermophilic enzyme belongs to class II of D-fructose-1.6-diphosphate aldolases. The thermophilic aldolase proved to be more stable than the mesophile. The substrate and 2-mercaptoethanol increased the stability of the thermophilic aldolase.

Key words: Thermostable, aldolase, characterization.

Introduction

Microorganisms growing at elevated temperatures possess a higher intrinsic thermostability than their mesophilic counterparts. There are several explanations for the thermostability of proteins. The theory of transferable protective factors (KOFFLER, 1957) has no experimental basis. AMELUNXEN and LINS (1968) verified the absence of stabilizing or labilizing factors. In vivo studies have revealed that the thermostability of some enzymes may stem from the interactions of the cellular components (HACHIMORI et al., 1974; WEDLER and HOFFMANN, 1974). Cofactors, substrates and monovalent or divalent ions may result in intracellular thermostability. According to other results, the reason for the thermostability may be the types and number of stabilizing forces such as H-bonds, apolar and ionic interactions operating in the enzyme molecule (CASS and STELLWAGEN, 1975; BRANDTS, 1967).

Thermophilic aldolase from *Bacillus stearothermophilus* has been investigated by JACK and HARRIS (1975). It is a dimeric Zn^{2+}-containing enzyme, with a half-life of about 30 min at 337 K. A gradual loss of activity was observed during the isolation, which could be prevented by adding Co^{2+} ion to the buffers. The Co^{2+}-enzyme cannot be inactivated to any detectable extent during 80 min at 337 K. Our study serves the comparative investigations between the aldolase of a facultative thermophile, *Bacillus sp.* JB-1 and two different types of mesophilic aldolases. The mesophilic enzymes were purified from *E. coli* and rabbit muscle, respectively. The thermophilic aldolase was characterized and the thermal stabilities of the three aldolases and the effects of various cations and substrate on the thermal stability were investigated.

Materials and Methods

Chemicals for general use were of analytical grade and were purchased from Reanal Factory of Laboratory Chemicals (Budapest, Hungary). Lysozyme was purchased from Serva Feinbiochemica (Heidelberg, FRG) and Sephacryl-200 and Sephadex gels from Pharmacia Fine Chemicals (Uppsala, Sweden).

Cultural conditions. A facultative thermophile, *Bacillus sp.* isolated at our Department and *E. coli* C-600 were maintained on 3% agar-agar. The thermophile strain was grown at 323 K with vigorous aeration in a medium containing 0.5% Yeast Extract Oxid, 1% glucose, 0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \times 7H_2O$, 0.01% $CaCl_2$. The pH of the medium was adjusted with 0.1 M NaOH to 7.5. Under these conditions the cells reached the end of the log phase of growth within 6 hours.

The *E. coli* cells were grown at 203 K with intensive shaking. The medium contained 1% Bacto Tryptone Difco, 0.5% Yeast Extract Oxoid, 0.5% glucose, and 0.5% NaCl in 0.1 M Tris/HCl buffer (pH 7.3). The cells reached the end of the log phase of growth within 16 hours.

The rabbit muscle aldolase was prepared and recrystallized three times according to TAYLOR et al. (1948). Its specific activity was 10 U/mg (in coupled reaction assay).

Enzyme assay was performed according to the method of JAGANATHAN et al. (1956) or with a coupled reaction assay in the presence of glycerol-3-phosphate dehydrogenase (GDH) and triosephosphate isomerase (TIM) (RUTTER et al., 1966). In the first case the reaction mixture contained 7 µmole hydrazine and 12 µmole D-furctose-1.6-diphosphate (FDP) in 0.1 M phosphate buffer (pH 7.5). Measurements were carried out at 240 nm, at 298 K.

The coupled reaction assays were performed in an assay mixture containing 0.1 mM NADH, 0.22 mM FDP, and 40 µg/ml GDH/TIM [the specific activity of GDH was approx. 130 U/mg in 0.2 M triethanolamine buffer (pH 7.9)], at 298 K.

Protein content of purified enzyme was determined spectrophotometrically. The absorption coefficient was found to be $A^{0.1\%} = 0.62$ for the thermophilic aldolase. The protein content for rabbit muscle aldolase was taken as $A^{0.1\%} = 0.75$ reported by FOX and DANDLIKER (1956).

Thermal inactivation was carried out in 0.1 M phosphate buffer, pH 7.5, as previously described by SZAJÁNI et al., (1970). 1 ml aliquots of enzyme solution with protein contents of 1.0—1.5 mg were incubated at the selected temperatures, then cooled and centrifuged.

Molecular weight was determined on Sephacryl-200 column (1.5×43 cm). Trypsin (Mw 23.800), hemoglobin (Mw 64.000), bovin serum albumin (Mw 68.000) and glucose oxidase (Mw 150.000) were used as molecular weight standards.

Results and discussion

PREPARATION OF BACTERIAL ALDOLASES.

Cells were cooled to 283 K and centrifuged. 50 g cell paste was washed twice with 0.1 M phosphate buffer (pH 7.5), containing 0.9% NaCl. Cells were lysed by the addition of lysozyme (1 mg/g cells). The suspension was stirred in a water-bath for two hours at 310 K, then overnight at 277 K. The lysis was performed too in french press (Aminco) at 20 000 psi cell pressure for 5 min. The degree of enzymatic lysis approached that of second method. Cell debris was removed by centrifugation at 20 000 g for 20 min. The precipitate was washed with the same buffer and the supernatants were collected. The crude extract was saturated up to 0.5 with solid $(NH_4)_2SO_4$ at 298 K, and the precipitate was removed by centrifugation. The supernatant was further saturated up to 0.75 with $(NH_4)_2SO_4$, the precipitate contained high aldolase activity. The protein was separated from the salt on a Sephadex G-25 column (2.5×20 cm) which was equilibrated with 0.02 M phosphate buffer (pH 7.5). The active fractions were collected and passed through a DEAE-cellulose column (1.5×11 cm) in 0.02 M phosphate buffer (pH. 7.5). The enzyme was eluted with the same buffer containing 0.2 M NaCl. The active fractions were collected and gel-filtered on a Sephadex G-150 column (2.5×30 cm) equilibrated with 0.1 M phos-

phate buffer (pH 7.5). The buffer contained 1 mM 2-mercaptoethanol in the case of the thermophilic enzyme, for without it the purified enzyme lost much of its activity during several hours.

CHARACTERIZATION OF THE THERMOPHILIC ALDOLASE.

The activity of the thermophilic aldolase has an optimum at 337 K and pH 7.5 (Fig. 1; 2). The specific activity was calculated as 0.32 unit/mg; the value of K_M was 7.3×10^{-4} M (Fig. 3).

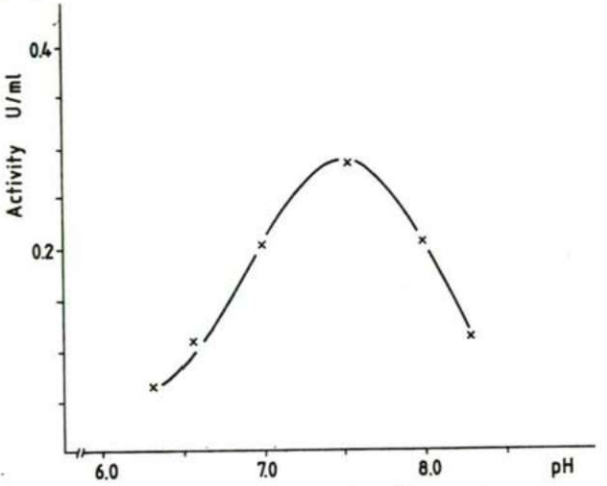

Fig. 1. Effect of pH on the activity of the thermophilic aldolase. The measurements were carried out in 0.1 M phosphate buffer with 0.1 mg/ml enzyme at 330 K.

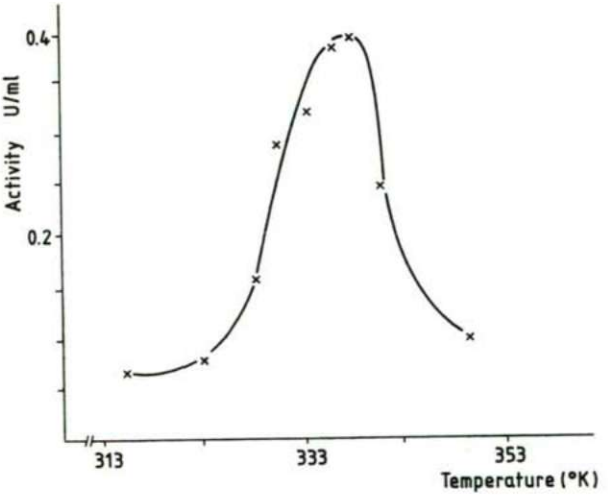

Fig. 2. Effect of temperature on the activity of the thermophilic aldolase. The measurements were carried out in 0.1 M phosphate buffer (pH 7.5) with 0.1 mg/ml enzyme.

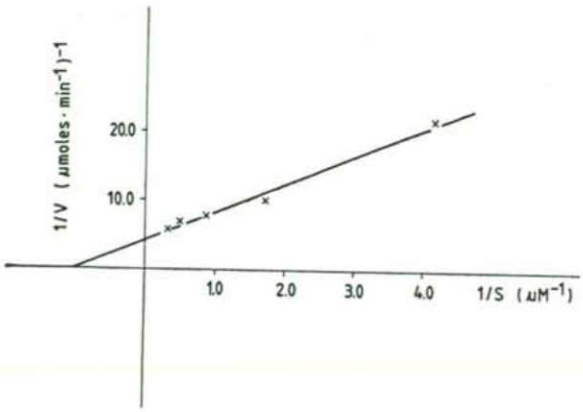

Fig. 3. Lineweaver — Burke plot for thermostable aldolase. Experiments were carried out in 0.2 M triethanolamine buffer (pH 7.9), with coupled reaction enzyme assay, at 298 K.

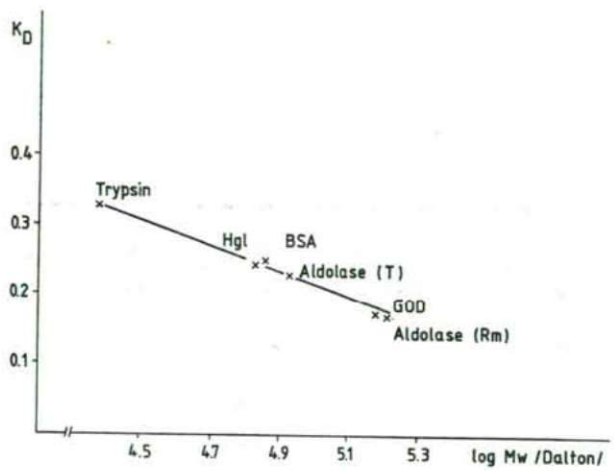

Fig. 4. Determination of molecular weight of the thermophilic aldolase on Sephacryl-200 column (1.5×43 cm.) The proteins were dissolved in 0.1 M Tris/HCl buffer (pH 7.5), containing 0.2 M NaCl. Standard proteins used were: trypsin, hemoglobin (Hgl), bovine serum albumin (BSA), rabbit muscle aldolase, glucose oxidase (GOD).

The molecular weight of the thermophilic enzyme was determined as 80. 300 dalton which is in good agreement with that of bacterial aldolases (Fig. 4).

The activity of the enzyme was not significantly enhanced by Zn^{2+}, Mg^{2+}, Na^{+} or K^{+} ions, but Co^{2+}, Ni^{2+}, Ca^{2+}, Mn^{2+} ions in 1.0 mM concentration increased the activity (Table I).

The effect of the Co^{2+} ion concentration on the activity of the thermophilic aldolase is shown in the Table II. The highest activity was measured at 1.0 mM $CoCl_2$. Considerable loss of activity occurred on the action of EDTA (Table III). This was

reversible, by the effect of 10 mM EDTA the enzyme was inhibited to 100%, but 80% of the activity was restored by adding 10 mM $CoCl_2$.

Thermal inactivation tests were carried out on the thermophilic and mesophilic aldolases.

Table 1. Effects of different cations on the activity of the thermophilic aldolase. Enzyme assays were performed in 0.1 M Tris/HCl buffer, pH 7.5. The enzyme concentration in the reaction mixture was 0.16 mg/ml.

Ions	Relative activity % Ion concentration	
	0.2 mM	1.0 mM
Ni^{2+}	89.4	141
Zn^{2+}	97.6	82.3
Ca^{2+}	105.7	135
Mg^{2+}	105.7	70.6
Mn^{2+}	81.3	117.7
Co^{2+}	181.3	370
Na^{+}	105.7	100
K^{+}	105.7	88.2

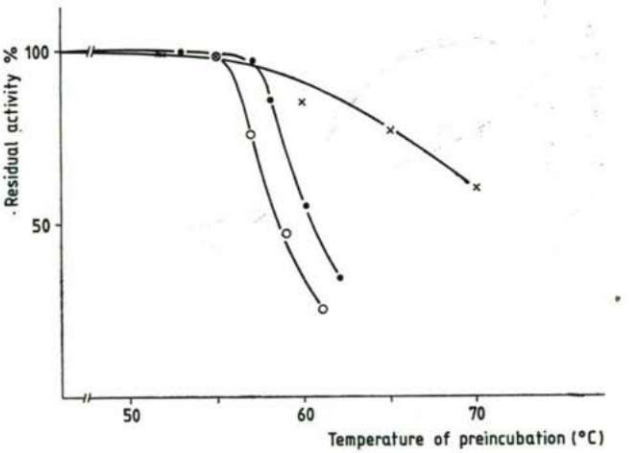

Fig. 5. Heat inactivation of *E. coli* aldolase (○——○) rabbit muscle aldolase (●——●) and the thermophilic aldolase (x——x). The samples were incubated at various temperatures for 10 minutes in 0.1 M phosphate buffer (pH 7.5). The protein content was 1.1 mg/ml.

A comparison of the thermal stabilities of *E. coli* aldolase, rabbit muscle aldolase and the thermophilic aldolase is shown in Fig. 5.

The aldolases from the mesophiles had similar thermal stabilities and their half-lives were comparable in the same range of temperature. When the protein

solution was incubated at 331 K in 0.1 M phosphate buffer (pH 7.5), the activity of muscle aldolase decreased to 50% during about 60 min, which is in good agreement with the data of ZÁVODSZKY et al. (1972).

Table 2. Effect of Co^{2+} ion concentration on the activity of thermophilic aldolase. The enzyme activity was measured in 0.1 M Tris/HCl buffer (pH 7.5) with an aldolase concentration of 0.16 mg/ml at 298 K.

Co^{2+} ion concentration M	Relative activity %
0.0	100
1×10^{-5}	155.1
2×10^{-5}	180.7
1×10^{-4}	251.8
2×10^{-4}	265.1
1×10^{-3}	346.1
2×10^{-3}	322.0

The thermal stability of the thermophilic aldolase highly saturated with FDP was modified by the substrate and the same effect was observed incubating the enzyme in 2-mercaptoethanol at 333 K (Fig. 6). These compounds markedly enhanced the stability of the enzyme.

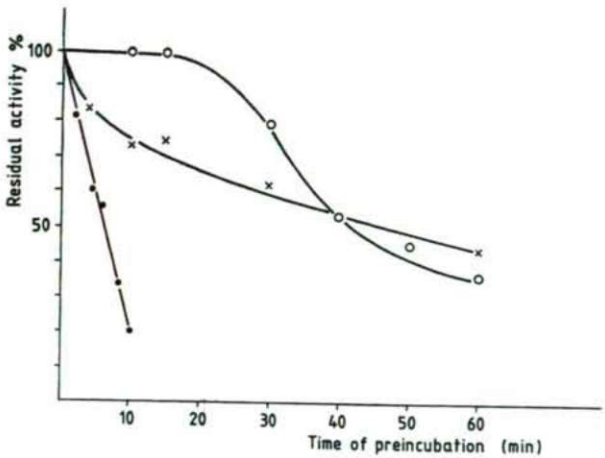

Fig. 6. Effects of substrate and 2-mercaptoethanol on the thermal stability of the thermophilic aldolase at 333 K. Heat treatment was performed in 0.1 M phosphate buffer (pH 7.5), containing 1.2 mM D-fructose-1.6-diphosphate (○——○) or 1 mM 2-mercaptoethanol (x——x). In the control samples the substrate and reducing compound were omitted from the protein solution (●——●). The enzyme concentration was 1.25 mg/ml.

To summarise the results, the thermophilic aldolase isolated from a facultative thermophile, *Bacillus sp.*, has a pH optimum of 7.5 at 337 K, the optimum temperature. Co^{2+} ions increased the activity. The aldolase was inhibited by EDTA. The ion

Table 3. Effect of EDTA on the enzymatic activity of the thermophilic aldolase. The activity was measured in 0.1 M Tris/HCl buffer (pH. 7.5) at 298 K. The enzyme concentration was 0.16 mg/ml.

Final molar of EDTA M	Relative activity %
0.0	100
3.5×10^{-4}	73.5
5.5×10^{-4}	48.6
1.0×10^{-3}	30.1
1.0×10^{-2}	0.0

dependence of the enzyme activity suggests that the thermophilic aldolase belongs to class II of D-fructose-1.6-diphosphate aldolases. Although this enzyme is similar to the aldolase of *Bacillus stearothermophilus* reported by JACK and HARRIS (1975), which also shows a high affinity towards Co^{2+} ions, addition of Zn^{2+} ions did not increase the activity in our case. The thermal stability of the thermophilic enzyme was higher than that of the mesophiles. The thermal stabilities of *E. coli* aldolase and muscle aldolase did not differ significantly from each other. The thermophilic enzyme requires thiol compounds for stability, whereas the aldolase from *E. coli* has no such requirement (BALDWIN et al., 1978). The stabilizing effect of the thermophilic enzyme has been observed. A general assumption concerning the thermal stability is that the substrate and thiol compounds conserve or convert the enzyme to a thermally stable and active conformation.

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