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ABSTRACT

THOMPSON, P. J. (University of Nebraska, Lincoln) AND T. L. THOMPSON. Some characteristics of a purified heat-stable aldolase. J. Bacteriol. **84**:694–700. 1962—Aldolase from a thermophilic strain of bacteria was obtained in a state of high purity. Heat studies of purified aldolases from cells cultivated at 45 and 65 C showed them equally stable at 70 C for 1 hr. Metal-ion and chelate studies indicated that thermal aldolase is metal ion-independent. Carboxypeptidase did not alter activity or specificity. The enzyme was specific for fructose-1,6-diphosphate. Hydrazine was found inhibitory in the assay procedure. The inhibition was independent of pH over the range of H^+ concentrations tested and was reversed by dialysis against water.

Much information about the nature of thermophily has been obtained through investigation of various reconstituted enzyme systems of thermophilic bacteria. With few exceptions, the enzymes of thermophilic bacteria are more refractory to heat than their mesophilic counterparts, using, as a criterion of stability, catalytic activity before and after heating (Marsh and Militzer, 1952; Militzer and Burns, 1954; Militzer et al., 1949).

Initial studies of the aldolase from a thermophile were made by Thompson (1950). He showed that aldolase of yeast or muscle was completely inactivated within 5 min at 65 C, whereas thermal aldolase had lost less than 1% of its activity after 1 hr at the same temperature. Beindorff (1952), Snyder (1953), and Thompson (1958) have verified these findings. In all of these experiments, aldolase was used in various states of purity.

This study was undertaken with the purpose of purifying thermal aldolase to a degree that would exclude any possible effects of contaminating material on thermal-inactivation data. A reproducible scheme for purification of bacterial aldolase as well as some characteristics of the enzyme are presented.

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MATERIALS AND METHODS

Bacillus stearothermophilus NCA 2184 is a stenothermophilic bacterium with a growth range of 45 to 65 C. Cultures were maintained on 2% Trypticase Agar slants and transferred weekly. Mass cultures were prepared by inoculating 1-liter Trypticase Broth shake cultures into 15-liter volumes of heated 2% Trypticase Broth. After vigorous aeration of the medium for 18 hr, the cells were removed by centrifugation and stored as a paste at −7 C.

Purification of aldolase. Except where indicated, the procedure was carried out at 25 C. A 200-g amount of cell paste was washed once with 400 ml of 0.9% NaCl solution. The organisms were resuspended in 200 ml of 0.9% NaCl solution to which 150 mg of crystalline lysozyme were added. The suspension was incubated for 2 hr at 37 C, then overnight at 4 C. Debris was removed by centrifugation at 20,000 × *g* for 20 min, and the sediment and supernatant fluid were saved. The sediment was washed once with 50 ml of 0.9% saline and the wash solution was added to the original supernatant fluid. The combined supernatant fluid was dialyzed to remove NaCl and brought to 0.5 saturation with $(NH_4)_2SO_4$ (pH 7.2). The resulting precipitate was removed by centrifugation and discarded. The concentration of $(NH_4)_2SO_4$ in the supernatant fluid was raised to 0.72 saturation, and the precipitate, which assayed high in aldolase, was removed, dissolved in demineralized water, and dialyzed against demineralized water for 48 hr at 4 C. The dialyzed protein solution was clarified by centrifugation and adjusted to pH 6.0 with 0.1 M acetic acid. To this solution (about 15 mg of protein per ml) were added 10 g of diethylaminoethyl (DEAE)-cellulose, which

had been adjusted to pH 6.0 with 0.02 M sodium phosphate buffer. The mixture was allowed to stand for 15 min, and was then centrifuged at 12,000 × *g* for 5 min. The supernatant fluid containing the enzyme was then dialyzed against demineralized water overnight at 4 C.

A column (3 5 by 10 cm) of DEAE-cellulose was adjusted to pH 8.0 by passing through it 0.005 M sodium phosphate buffer until the effluent remained at pH 8.0. The enzyme preparation was adjusted to pH 8.0 with 1 N NaOH and policed onto the column. After washing with 1 liter of 0.005 M phosphate buffer (pH 8.0) and with 1 liter of 0.005 M phosphate buffer (pH 7.0), the aldolase was eluted with 0.05 M phosphate buffer (pH 6.0). Effluent fractions assaying high in aldolase were pooled, dialyzed against demineralized water, and recycled through the cellulose adsorbent. After one more careful dialysis against demineralized water, the enzyme solution was lyophilized and stored as a white powder over $CaCl_2$ at 4 C. The usual recovery was about 15% of the total aldolase assayed in the lysozyme extract. Since emphasis was on purity, only limited attempts were made to mprove recovery.

Similar recoveries of purified aldolase were obtained from cells cultivated at 45 C. The aldolase content of the cells appeared to be independent of growth temperature, assuming that all of the enzyme was released from the cells by the lysozyme treatment.

Assay of aldolase activity. Hexose cleavage was measured by the colorimetric method of Dounce, Barnett, and Beyer (1950), using an incubation temperature of 58 C for the bacterial aldolase and 37 C for the muscle aldolase. Rabbit-muscle aldolase was a 5 × recrystallized suspension purchased from Nutritional Biochemical Corp., Cleveland, Ohio. A standard curve was constructed by substituting the sodium salts of the triose phosphoric acids into the usual assay procedure. The trioses had been converted to the free form from their cyclohexylammonium derivatives according to the instructions received from the manufacturer (Sigma Chemical Co., St. Louis, Mo.).

Some of the results of the chemical-assay method were substantiated with the optical-enzymatic method of Warburg and Christian (1943). Owing to the lability of the glyceraldehyde-phosphate dehydrogenase, which is added

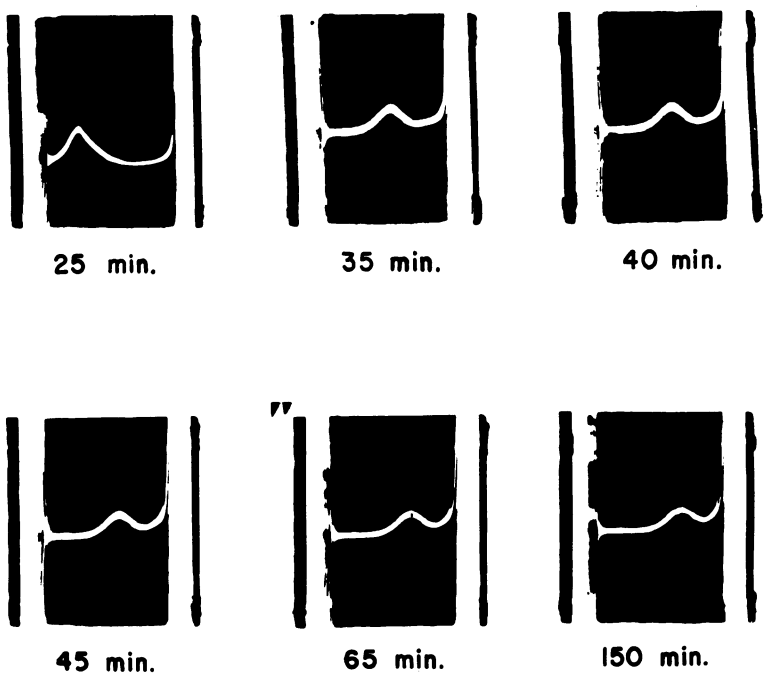

FIG. 1. *Sedimentation pattern of thermal aldolase. The enzyme was dissolved in 0.1 M tris buffer (pH 9.0). Sedimentation was from left to right at 58,000 rev/min with rotor temperature at 25 C.*

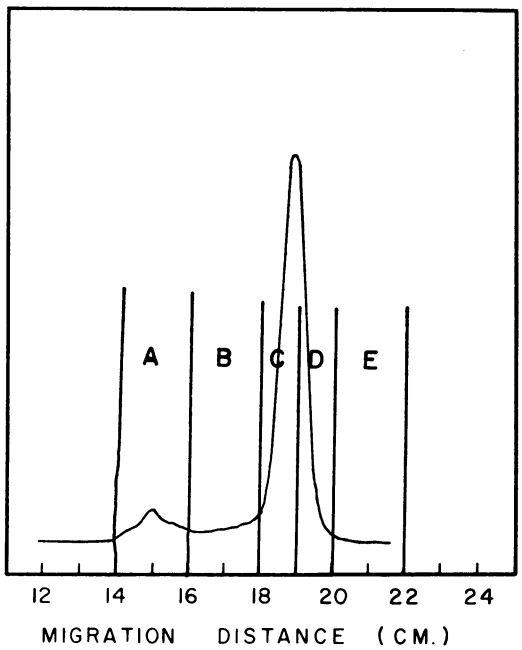

FIG. 2. *Electrophoretic diagram of thermal aldolase. Conditions were: diethylbarbiturate buffer (pH 8.6), ionic strength, 0.075; 18 hr at 180 VDC. Segments* (A, B, C, D, *and* E) *were cut from strip and assayed for aldolase. The moles of triose formed* $\times 10^{-8}$ *were as follows for each segment:* A (*origin*), *0;* B, *0.5;* C, *12.5;* D, *8.3;* E, *0.*

to the incubation mixture, this assay was conducted at room temperature. At this temperature, thermal aldolase activity was of such a low order that approximately ten times as much enzyme as used in assays conducted at 58 C was required to show an optimal rate of diphosphopyridine nucleotide reduction.

Electrophoresis. Hanging-strip electrophoresis was carried out in a Spinco electrophoresis cell. The protein boundaries were detected with bromophenol blue and scanned on a Spinco densitometer. Examination of the strips for enzyme activity was done as follows. Duplicate unstained strips were placed along side the stained strips; segments of paper corresponding to regions of heavy staining, as well as clear areas, were cut from the unstained strip and extracted with demineralized water; the extracts were then carried through the assay procedure.

Enzymatic hydrolysis. Carboxypeptidase (Nutritional Biochemical Corp., Cleveland, Ohio) was used in hydrolysis studies of the bacterial aldolase. The exopeptidase was standardized against carbobenzoxy-L-glycylphenyalanine. The rate of hydrolysis was followed according to the copper-salt method of Spies and Chambers (1951).

TABLE 1. *Effect of divalent metal ions, chelating agents, cysteine, and peroxide on thermal aldolase*

Reagent tested	Final concn (molarity)*	Aldolase activity remaining
	M	%
None	—	100
$ZnCl_2$	1.6×10^{-3}	75
$CoCl_2$	1.6×10^{-3}	100
$MgCl_2$	1.6×10^{-3}	100
$SnCl_2$	1.6×10^{-3}	55
$CuSO_4$	1.6×10^{-3}	0
$FeSO_4$	1.6×10^{-3}	14
$MnCl_2$	1.6×10^{-3}	10
$NiCl_2$	1.6×10^{-3}	0
$Pb(NO_3)_2$	1.6×10^{-3}	0
$HgCl_2$	1.6×10^{-3}	0
Cysteine	1×10^{-4}–5×10^{-2}	100
H_2O_2	5×10^{-5}–5×10^{-3}	100
8-Hydroxyquinoline	1×10^{-5}–1×10^{-2}	100
2,2¹-dipyridyl	1×10^{-5}–1×10^{2}	100

* Metal salts were dissolved in 0.05 M tris-(hydroxymethyl)aminomethane buffer, pH 7.2. Final pH of incubation mixtures ranged from 7.0 to 7.5.

Heat studies. Thermal-inactivation tests were conducted by adding measured volumes of aldolase dissolved in demineralized water to 18-mm Pyrex tubes. Sufficient demineralized water was added to give final volumes of 0.5 ml. The tubes were then placed in a 70-C bath, removed at various time intervals, and assayed. Protein estimations were made according to Lowry et al. (1951).

RESULTS

All data were obtained using purified aldolase from cells grown at 65 C, unless otherwise stated.

Figure 1 represents the ultracentrifugal picture of purified bacterial aldolase. The Svedberg value for the enzyme is 3.58, uncorrected for viscosity and density of solvent. Careful examination of this figure will disclose a slight bulge in the descending slope of the sedimen-

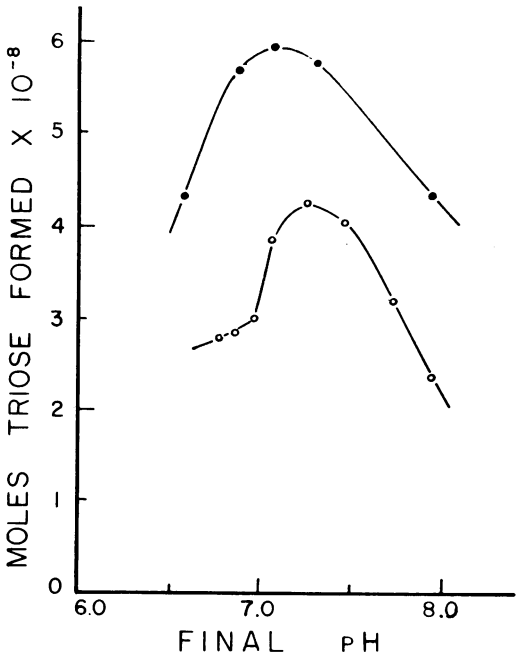

FIG. 3. *Effect of pH on hydrazine inhibition. Incubation mixtures contained: 0.5 ml of 0.1 M sodium hexosediphosphate (pH 6.9); 0.2 ml of 0.05 M borate buffer (pH 7.6 to 9.2); 0.1 ml of solution containing 8.0 μg of lyophilized enzyme. Total volume was 1.0 ml. Final pH of reaction mixtures was determined on duplicate unincubated samples.* ●, *no hydrazine;* ○, *with hydrazine.*

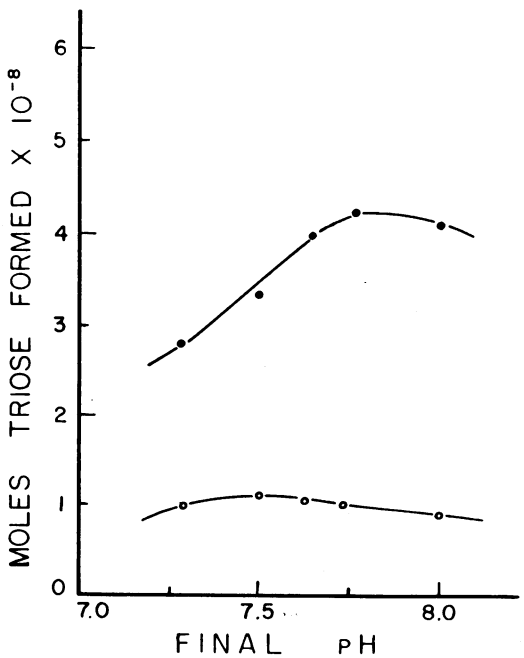

FIG. 4. *Effect of pH on hydrazine inhibition. Incubation mixtures same as Fig. 3 except 0.2 ml of 0.05 M phosphate buffer (pH 7.5 to 8.2) was substituted for borate buffer.* ●, *no hydrazine;* ○, *with hydrazine.*

tation pattern. This more rapidly sedimenting boundary may be due to triosephosphate isomerase, an impurity which was assayed in all aldolase preparations regardless of growth temperature. Concentrated solutions of aldolase were required to demonstrate the isomerase activity, since the isomerase assay was conducted at room temperature. Dilutions (20-fold) of concentrated solutions required to measure isomerase activity would suffice for suitable readings of aldolase activity in the colorimetric assay technique. Further steps taken to remove triose isomerase resulted in a net loss of aldolase with no measurable increase in specific activity.

That the same bacterial aldolase preparation is homogeneous by hanging-strip electrophoresis criteria may be due to a limiting dye-binding capacity of the trace contaminant (Fig. 2).

The purified aldolases from cells grown at 45 and 65 C were subjected to thermal studies. After exposure to 70 C for 60 min, both aldolases had lost about 5% of their original activity. When the thermal studies were repeated, using all fractions of the purification process, the same results were obtained. It is probable that no stabilizing or labilizing factors were removed during the purification.

During the purification of the aldolase, stimulation by cysteine, H_2O_2, 8-hydroxyquinoline, 2,2'-dipyridyl, and Co^{++} was noted. After the second passage through the cellulose exchanger, these effects disappeared. Table 1 summarizes the effects of various metal salts, chelating agents, cysteine, and peroxide on the purified aldolase. Under the conditions of aldolase assay, it is concluded that the purified bacterial aldolase has no metal requirements.

During kinetic studies of the purified aldolase, hydrazine, the trapping agent for glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, was omitted from the incubation mixture. A higher activity resulted. Since the efficiency of a trapping agent varies with the pH (Sibley and Lehninger, 1949), a range of H^+ concentra-

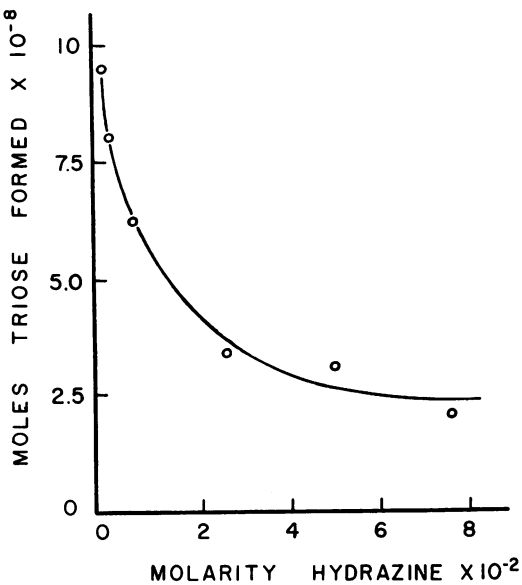

FIG. 5. *Hydrazine inhibition of thermal aldolase activity. Reaction mixtures contained 0.2 ml of diluted 1.0 M hydrazine (pH 7.2). Water was substituted for buffer. Otherwise, the mixture was the same as in Fig. 3. Final pH was 7.2.*

TABLE 2. *Effect of hydrazine on rabbit-muscle aldolase activity*

Final molar concn of hydrazine added to 5 μg of rabbit-muscle aldolase*	Moles triose formed × 10^{-5} × $15\ min^{-1}$ × $mg\ protein^{-1}$
0.0	9.2
0.0025	15.6
0.005	19.6
0.01	16.0
0.05	16.3
0.075	16.0
0.1	16.5

* Incubation temperature: 37 C. Final pH of reaction mixtures 7.2 to 7.4.

tions was tested, using phosphate and borate buffers (Fig. 3 and 4). These data indicate that the hydrazine inhibition is not a pH phenomenon, at least within the pH range tested.

The extent of hydrazine inhibition of bacterial aldolase is shown in Fig. 5. By comparison, rabbit-muscle aldolase activity is enhanced by hydrazine (Table 2). Since the same reagents were used for the assay of both aldolases, it was assumed that the apparent activity decrease

TABLE 3. *Reversibility of hydrazine inhibition of thermal aldolase activity*

Dialyzed sample*	Moles triose formed × 10^{-7} × $15\ min^{-1}$ × $mg\ protein^{-1}$
Aldolase vs. hydrazine	4.3
Aldolase vs. water (control)	23.1
Hydrazine-inhibited adolase vs. water	19.8
Control vs. water	24.0

* Dialysis was carried out against 0.5 M hydrazine (pH 7.2) in polyethylene containers at 4 C.

TABLE 4. *Effect of carbonyl reagents on thermal aldolase activity*

Reagent*	Final molarity	Moles triose formed × 10^{-7} × $15\ min^{-1}$ × $mg\ protein^{-1}$
None	—	30
NaCN	5×10^{-3}	16
$NaHSO_3$	5×10^{-3}	9
H_2NOH	5×10^{-3}	9

* Final pH of all reagents tested was 7.2.

was not due to hydrazine interference with color development.

Reversibility of the hydrazine effect was demonstrated in the following way. Two samples of the bacterial aldolase were dialyzed overnight, one against hydrazine, the other against demineralized water. After dialysis, concentrations of both solutions were equalized on the basis of protein. The aldolase activity of the solutions was determined (Table 3). Both solutions were then returned to the dialyzing membranes and dialyzed overnight against water. The protein concentrations were then equalized and assayed for aldolase (Table 3). Approximately 83% of the activity was recovered if the control activity was set at 100%.

Other carbonyl reagents were tested in regard to possible inhibitory effects on the bacterial aldolase (Table 4).

Enzymatic hydrolysis experiments revealed that the activity of the bacterial aldolase is abolished by ficin, but unaffected by trypsin. Exposure to carboxypeptidase for periods up to 2 hr resulted in no alteration of activity and no

measurable hydrolysis of enzyme protein. These results differ from those of Drechsler, Boyer, and Kowalsky (1959), who noted a 90% loss of aldol cleavage activity of muscle aldolase within 23 min after exposure to carboxypeptidase.

Measuring the cleavage reaction, the bacterial aldolase appears specific for fructose-1,6-diphosphate. Fructose-1-phosphate is not a substrate for the enzyme. Similarly, fructose-6-phosphate cleavage was not catalyzed by the bacterial aldolase, although initial tests were confounded by the presence of the diphosphorylated hexose in commercial preparations. Repeated recrystallizations of the barium salt did not effect sufficient purification. The purification method of Khym and Cohn (1953) finally settled this problem.

DISCUSSION

Investigations of alpha-amylase by Campbell (1955) indicated that a direct relationship exists between heat stability of some enzymes and the temperature at which the containing cells are incubated. Brown, Militzer, and Georgi (1957) and Zobel (1957), who worked with inorganic pyrophosphatase and malic dehydrogenase, respectively, found this same heat stability-growth temperature dependence. Results given in this paper do not bear out the findings stated above; however, this may be due to an inherent heat stability of bacterial aldolase.

Purified thermal aldolase is not inhibited by metal-chelating agents. According to Rutter et al. (1960), it is a type I aldolase and is classified with muscle aldolase, which also is metal ion-independent. Thermal aldolase differs from the aldolases of *Aspergillus niger*, *Saccharomyces cerevisiae*, *Escherichia coli*, *Lactobacillus bifidus*, *Brucella suis*, *Mycobacterium tuberculosis*, and *Clostridium perfringens*, which are type II aldolases, i.e., metal ion-dependent.

The inhibition of thermal aldolase by Pb^{++}, Cu^{++}, and Hg^{++} suggests that free sulfhydryl groups are essential for full catalytic potency. The difference in response to the action of carboxypeptidase by the bacterial and muscle aldolases implies a difference in terminal amino acids of the two enzymes. Data by Richards and Rutter (1961) demonstrate that the purified yeast aldolase is also unaffected by carboxypeptidase. Yeast and muscle aldolases do, however, show a satisfactory agreement in regard to their rate of fructose-1-phosphate turnover, which is not shared by thermal aldolase. This difference in substrate specificity may reflect distinctive sites on the thermal aldolase, especially when the hydrazine inhibition is taken into consideration. Since hydrazine would not react with any known essential groups on the enzyme protein, it is proposed that the observed inhibition of aldol cleavage is due to the blocking of active sites by the hydrazine-bonded cleavage products. This hypothesis is in conformity with the extent of hydrazine inhibition, as well as the reversibility of the inhibition.

ACKNOWLEDGMENTS

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