

Purification and Characterization of a Repressible Alkaline Phosphatase from *Thermus aquaticus**

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A repressible alkaline phosphatase has been isolated from the extreme bacterial thermophile, *Thermus aquaticus*. The enzyme can be derepressed more than 1,000-fold by starving the cells for phosphate. In derepressed cells, nearly 6% of the total protein in a cell-free enzyme preparation is alkaline phosphatase. The enzyme was purified to homogeneity as judged by disc acrylamide electrophoresis and sodium dodecyl sulfate electrophoresis. By sucrose gradient centrifugation it was established that the enzyme has an approximate molecular weight of 143,000 and consists of three subunits, each with a molecular weight of 51,000. Tris buffer stimulates the activity of the enzyme, which has a pH optimum of 9.2. The enzyme has a broad temperature range with an optimum of 75–80°. The enzyme catalyzes the hydrolysis of a wide variety of phosphorylated compounds as do many of the mesophilic alkaline phosphatases. The Michaelis constant (K_m) for the enzyme is 8.0×10^{-4} M. Amino acid analysis of the protein revealed little in the amino acid composition to separate it from other mesophilic enzymes which have been previously studied.

Thermus aquaticus is an extreme bacterial thermophile isolated from thermal springs in Yellowstone National Park (1). This species is especially interesting because it can be grown in defined media at temperatures up to 80°. It is, thus, suitable for comparing the physiological processes of a microorganism which lives at a biological extreme to similar processes which have been more extensively studied in mesophilic bacteria such as *Escherichia coli* and *Salmonella typhimurium*.

Detailed studies have been initiated on many thermophilic proteins such as α -amylase (2), aldolase (3), β -galactosidase (4), and thermolysin (5) in order to determine some of the parameters which account for the stability of thermophilic enzymes at high temperatures. Unfortunately, the studies so far have not revealed significant physical or chemical differences between thermophilic proteins and their mesophilic counterparts. It would seem that any differences between mesophilic and thermophilic proteins are subtle ones which can be fully elucidated only by determining the primary structure of the proteins by amino acid sequencing and their 3-dimensional structures by x-ray crystallography (6). The present report, concentrating on physiological aspects of thermophilic enzyme functioning, describes the derepression, purification, and characterization of an alkaline phosphatase which has a temperature optimum of 75–80°. It compares some of the properties of this enzyme with those of other alkaline phosphatases from both prokaryotic and eukaryotic microorganisms such as *E. coli* (7), *Bacillus licheniformis* (8), *Micrococ-*

cus sodonensis (9), *Neurospora crassa* (10), and *Aspergillus nidulans* (11).

EXPERIMENTAL PROCEDURES

Strain—*Thermus aquaticus* YT-1 used in these experiments was supplied by Dr. Paul Ray, Burroughs Wellcome.

Culture Medium—Cells were grown in a defined mineral salts medium containing 0.3% glutamic acid (which served as both a carbon and nitrogen source) which was supplemented with biotin and thiamin (0.1 mg/liter each) and nicotinic acid (0.05 mg/liter). The salts included in 1 liter of medium were: nitrilotriacetic acid, 100 mg; $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 60 mg; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 100 mg; NaCl, 8 mg; KNO_3 , 103 mg; NaNO_3 , 689 mg; ZnSO_4 , 5 mg; H_3BO_3 , 5 mg; CuSO_4 , 0.16 mg; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.25 mg; CoCl_2 , 0.4 mg; FeCl_3 , 0.28 mg; and $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 22 mg. Phosphate was added in limiting amounts using sodium glycerol phosphate which allowed for derepression of the alkaline phosphatase. The pH of the medium was adjusted to 8.0 with NaOH.

Growth Conditions—Cells were initially grown in 500-ml Erlenmeyer flasks at 75° in a New Brunswick water bath shaker. When the cultures reached a density of approximately 170 Klett units, 1 liter of these cells was transferred to 16-liter carboys and these were placed in hot air incubators. In place of shaking, sterile air was aerated, through the cultures and the temperature was maintained at 75°. The cells were allowed to grow for 24 hours before they were collected with a Sharples continuous flow centrifuge.

Preparation of Enzyme Extract—Subsequent to collection, the cells were suspended in 0.01 M Tris buffer (pH 8.0) containing 1 mM CaCl_2 . The cells were ruptured by a Branson 20-kc magnetostrictive ultrasonic oscillator, operated at 3.5 A for 20 s. The extracts were then spun in a Sorvall RC-2B centrifuge at $27,000 \times g$ for 10 min. The supernatant fluid served as the crude extract.

Enzyme Assay—Phosphatase activity was measured by observing the absorbance at 410 nm on a Gilford recording spectrophotometer of the *p*-nitrophenol which was released from *p*-nitrophenyl phosphate by the enzyme at 75°. The assay mixture contained *p*-nitrophenyl phosphate (1 mM), CaCl_2 (1 mM), and Tris buffer (0.5 M, pH 9.0) in a

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final volume of 0.5 ml. The reaction was stopped 10 min later by the addition of 0.5 ml of 0.4 M NaOH. One unit of enzyme activity is defined as the release of 1 μ mol of nitrophenol/min by the enzyme.

Protein Determinations—Protein concentrations were determined by the method developed by Lowry *et al.* (12).

Disc Gel Electrophoresis—Disc gels were prepared according to the method of Davis (13). The separating gel (8.0 \times 0.5 cm) contained 7% acrylamide, whereas the spacer gel (1.5 \times 0.5 cm) contained 2% acrylamide. Electrophoresis, in a Canalco model 66 electrophoresis bath, was carried out at room temperature in 2.5 mM Tris/1.9 mM glycine buffer, pH 9.5, for 2.5 hours at 2 mA. The gels were stained with 0.1% Coomassie brilliant blue in 7% acetic acid and subsequently destained in 7% acetic acid.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—Sodium dodecyl sulfate gels were prepared and run according to the method of Weber and Osborn (14). In these experiments four peptide markers were used as standards; phosphorylase A ($M_r = 94,000$), serum albumin ($M_r = 67,000$), ovalbumin ($M_r = 43,000$), and chymotrypsinogen ($M_r = 25,700$).

Sedimentation Coefficient—Purified alkaline phosphatase (40 μ g) and serum albumin (2.7 mg) were layered on a linear sucrose gradient of 6 to 25%. The gradient was also made up of 0.02 M Tris buffer, pH 8, and 1 mM CaCl₂. Centrifugation was performed using a Spinco SW 50L rotor in a preparative Beckman ultracentrifuge at 38,000 rpm for 17 hours at 3°. Fractions were collected from the bottom and assayed for serum albumin by absorbance at 280 nm and alkaline phosphatase by the liberation of nitrophenol. The sedimentation coefficient and approximate molecular weight for alkaline phosphatase were determined by the method of Martin and Ames (15).

Inorganic Phosphate—Inorganic phosphate was determined by the method of Fiske and SubbaRow (16).

Amino Acid Analysis—Amino analyses were performed according to Spackman *et al.* (17) on a Durrums D-500 amino acid analyzer. Acid hydrolysis (24, 48, 72, and 96 hours) of protein samples, containing 0.12 mg/tube in 1 ml, was carried out in duplicate with 6 N HCl *in vacuo* at 110°. Cysteine and methionine were analyzed as cysteic acid and methionine sulfone, respectively, after oxidation with performic acid (18). Serine and threonine values were obtained from extrapolated values to zero time of hydrolysis according to Moore and Stein (19). The tryptophan content was determined spectrophotometrically by the method of Edelhoch (20).

Chemicals—DEAE-cellulose was purchased from Gallard-Schlesinger Chemical Corp. The phosphocellulose was Whatman grade P-1 prepared as described by Loeb (21). The hydroxyapatite was Bio-Gel HTP (Bio-Rad Laboratories). Sephadex G-25 (Pharmacia) was used for desalting the enzyme. The organophosphates used as substrates were purchased from Kodak Chemical. Other chemicals were reagent grade from Fisher and Sigma.

RESULTS

Purification of Enzyme

The relative levels of alkaline phosphatase were examined in media containing various concentrations of inorganic and organic phosphate. The level of the enzyme varied over 1,000-fold depending on the amount of phosphate added to the growth media (Table I). Sodium glycerol phosphate (4×10^{-5} M) gave the best derepression with a reasonable cellular yield.

For the purification of the enzyme 32 liters of cells were harvested and processed as described under "Experimental Procedures." The extract was placed on a DEAE-cellulose column which had been equilibrated with 10 mM Tris buffer, pH 8, containing 1 mM CaCl₂. The column used was a Pharmacia K50/60 with flow adaptors and had a set bed volume of 520 ml. A linear 1400-ml gradient (50 ml/hour) was used from 0 to 0.25 M NaCl in 10 mM Tris buffer, pH 8, containing 1 mM CaCl₂. The column was run at 4° as were all the subsequent columns and dialyses. The enzyme was eluted as a peak of activity at approximately 0.08 M NaCl. The pooled fractions were then dialyzed against 10 mM potassium phosphate, pH 8, containing 1 mM CaCl₂. The enzyme was then

TABLE I

Effect of phosphate limitation on levels of alkaline phosphatase in *Thermus aquaticus*

The cells were grown in 500-ml Erlenmeyer flasks at 75° in a New Brunswick water bath shaker for 30 hours. The cell densities varied from 120 to 190 Klett units depending upon the relative amount of phosphate added to the individual flasks.

Amendment		Specific activity units/mg
Inorganic phosphate	8×10^{-3} M	0.06
	4×10^{-3} M	0.18
	2×10^{-4} M	2.94
	4×10^{-5} M	21.70
Sodium glycerol phosphate	2×10^{-4} M	63.8
	4×10^{-5} M	91.4

placed on a phosphocellulose column with a set bed volume of 102 ml packed in a K26/40 Pharmacia column. A 500-ml linear gradient of NaCl, 0 to 0.25 M in phosphate buffer, 10 mM, pH 8, was used to elute the enzyme. The enzyme chromatographed as a single peak at approximately 0.1 M NaCl. The pooled fractions were dialyzed overnight against 10 mM potassium phosphate, pH 8, containing 1 mM CaCl₂. The enzyme was then placed on a hydroxyapatite column which had a set bed volume of 15 ml in a K15/30 Pharmacia column. The enzyme was eluted with a 200-ml linear gradient of potassium phosphate buffer, 0.01 to 0.25 M, pH 8. The enzyme was eluted at 0.07 M potassium phosphate as a single peak of activity with most of the protein corresponding to it. A summary of the purification procedure is shown in Table II. The enzyme, after purification, was stable and was stored frozen at -10° without appreciable loss of activity for at least a period of 2 months.

Properties of Purified Alkaline Phosphatase

Criteria of Homogeneity—The purified alkaline phosphatase was judged as pure because symmetrical peaks of activity and protein were obtained when the purified enzyme was rechromatographed on hydroxyapatite (Fig. 1). These data were confirmed by the behavior of the enzyme during electrophoresis on acrylamide disc gels. The results from disc gels can be seen in Fig. 2A. A single protein band was evident when the gels were stained with Coomassie blue, and this band was coincident with the phosphatase activity when the gels were sliced and assayed for catalytic activity. In addition, when the enzyme was denatured and run on a sodium dodecyl sulfate gel, only a single protein band could be seen (Fig. 2B).

Molecular Weight—The approximate molecular weight of the purified enzyme was determined by the method of Martin and Ames (15) and is described in detail under "Experimental Procedures." When the alkaline phosphatase was run in a sucrose gradient in the presence of either 1 or 10 mM CaCl₂ and with serum albumin as a protein marker, two peaks of phosphatase activity were seen (Fig. 3). Assuming that serum albumin has a molecular weight of 68,000 and is 5.7 S, then the two peaks of activity correspond to 9.3 S and 12.9 S. This by the method of Martin and Ames (15) translates into approximate molecular weights of 143,000 and 233,000. If the experiment is performed in the absence of CaCl₂ on a purified enzyme fraction which has been dialyzed and devoid of CaCl₂, one peak of activity can be seen which sediments slightly faster than β -galactosidase (M_r wt = 520,000).

TABLE II
Summary of purification procedure

Fraction	Volume	Total alkaline phosphatase activity ^a	Total protein ^b	Yield of activity	Specific activity	Purification
	ml	units	mg	%	units/mg	fold
Crude	248	213,000	2,718		78.4	
DEAE-cellulose	500	60,000	220	28	275	3.5
Phosphocellulose	200	61,000	60	28	1,023	13.0
Hydroxyapatite	35	39,700	30.1	19	1,320	16.9

^a One unit of alkaline phosphatase equals the release of 1 μ mol of nitrophenol/min at 75°.

^b Protein concentrations were determined by the method of Lowry *et al.* (12).

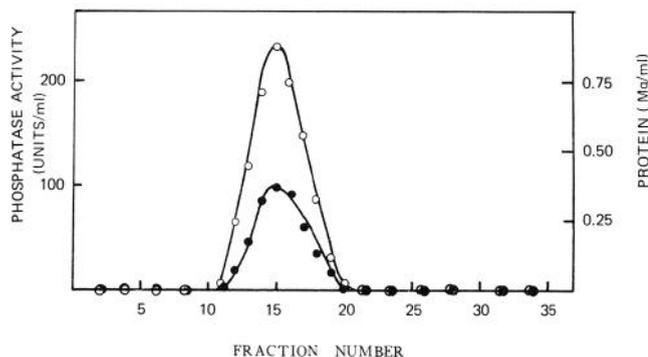


FIG. 1. Refractionation of purified enzyme on hydroxyapatite. Purified alkaline phosphatase (8 mg) was placed on a hydroxyapatite column and eluted with a linear gradient of phosphate buffer (0.01 M to 0.25 M). Each fraction contained 7 ml and each was assayed for protein (●) and for phosphatase activity (○).

In contrast to these data the molecular weight of the denatured enzyme was determined by sodium dodecyl sulfate gels to be approximately 51,000 (Fig. 4).

Temperature Optimum—Since *Thermus aquaticus* is an extreme thermophile which can grow at temperatures up to 80°, it was instructive to examine the effect of temperature on the catalytic properties of the enzyme. An experiment was performed where the enzyme was assayed at various temperatures (Fig. 5). The temperature optimum was 75–80°, although the enzyme showed activity over an unusually broad range of temperature.

Effect of pH and Buffer Concentration—The activity of the purified alkaline phosphatase was assayed at various pH values at 75°. The activity was considerably higher in Tris buffer than in glycine/NaOH buffer (Fig. 6). The pH optimum was approximately 9.2. When the enzyme was assayed in the presence of various concentrations of buffer, the activity was increased with the addition of Tris and decreased with the addition of glycine/NaOH (Fig. 7).

Michaelis Constant—The Michaelis constant (K_m) was determined for *p*-nitrophenyl at 75°. The K_m , 8.0×10^{-4} M, was determined graphically by the method of Lineweaver and Burk (22) and can be seen in Fig. 8. This value is slightly larger than the K_m values observed in *Escherichia coli* (7), *Micrococcus sodonensis* (9), and *Bacillus licheniformis* (8).

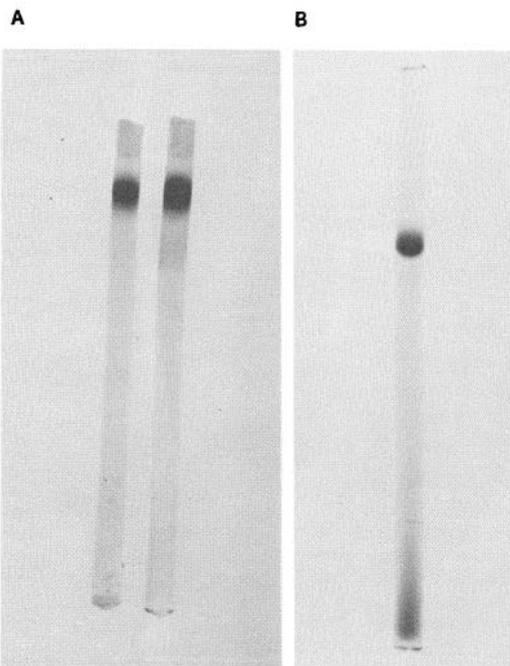


FIG. 2. Gel electrophoresis of purified enzyme. A, represents polyacrylamide gel electrophoresis of purified enzyme. Purified alkaline phosphatase (10 and 20 μ g) was applied to the gels and electrophoresis was carried out at room temperature in 2.5 mM Tris/1.9 mM glycine buffer, pH 9.5, for 2.5 hours. Electrophoresis at pH 7.0 and 8.4 gave the same results. B, represents sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified enzyme. Alkaline phosphatase (10 μ g) was run on sodium dodecyl sulfate-polyacrylamide gels as described under "Experimental Procedures." As can be seen only a single band of protein could be identified when the gels were stained with Coomassie brilliant blue.

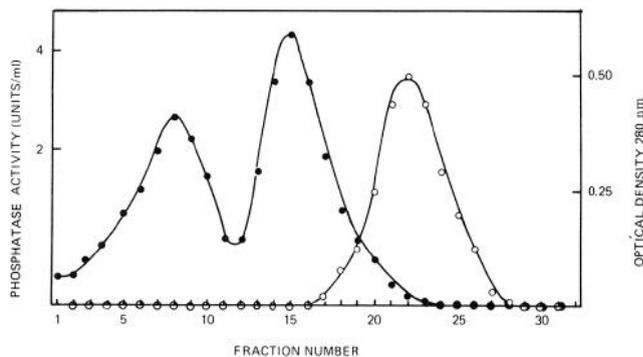


FIG. 3. Sedimentation coefficient by sucrose gradient centrifugation. Purified alkaline phosphatase (60 μ g) and serum albumin (2.7 mg) were layered on a linear sucrose gradient of 6 to 25% in 0.02 M Tris, pH 8, containing 1 mM CaCl_2 . Centrifugation was performed as described under "Experimental Procedures." Fractions of 15 ml were collected from the bottom and assayed for serum albumin by absorbance at 280 nm (○) and alkaline phosphatase by the appearance of nitrophenol (●).

Substrate Specificity—The relative rate of release of orthophosphate by the purified enzyme was measured for different phosphorylated substrates (Table III). As is the case with most other alkaline phosphatases which have been studied, the enzyme has the ability to hydrolyze a wide variety of phosphorylated compounds. It was, however, unable to hydrolyze pyrophosphate.

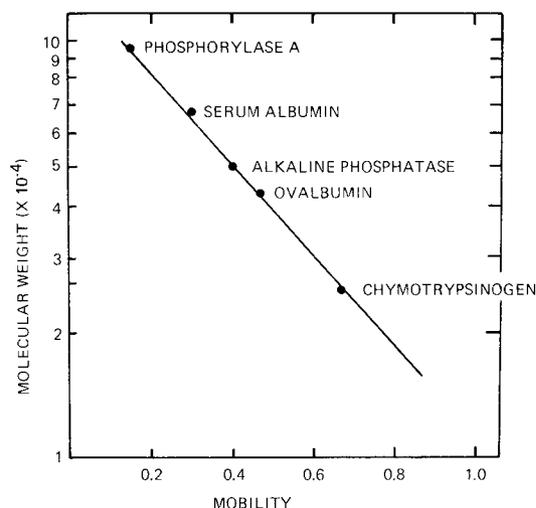


FIG. 4. Molecular weight estimation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Purified alkaline phosphatase and standard proteins of known subunit molecular weight were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were run as described under "Experimental Procedures" and stained for protein. The molecular weight of alkaline phosphatase was calculated by the method of Weber and Osborn (14).

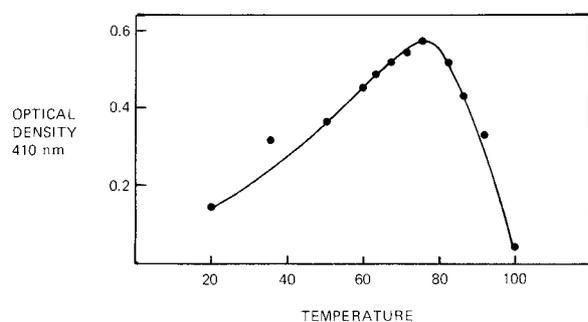


FIG. 5. Effect of temperature on the activity of purified alkaline phosphatase. Alkaline phosphatase (0.002 $\mu\text{g}/\text{assay tube}$) was incubated for 10 min at different temperatures. The reaction mixture contained 0.5 M Tris buffer, pH 9, 1 mM CaCl_2 , and 1 mM *p*-nitrophenyl phosphate. Since there is a small amount of nonenzymatic hydrolysis, especially at the higher temperatures a control without enzyme was run at each temperature.

Amino Acid Composition—The results, expressed as residues of amino acids per $51,000 \times g$ of protein, are summarized in Table IV. The protein does not appear to be particularly high or low in any 1 residue and contains two half-cystines/subunit. The proline content was relatively normal and much lower than the proline content reported for a thermophilic α -amylase by Campbell and Manning (2).

DISCUSSION

A repressible alkaline phosphatase from the extreme thermophile *T. aquaticus* has been purified to homogeneity as judged by polyacrylamide disc gel electrophoresis and sodium dodecyl sulfate disc gel electrophoresis. The enzyme is made up of subunits of a molecular weight of 51,000, and it appears to exist in two forms, each made up of multiples of the subunits. The question of whether the two forms of the enzyme are important physiologically or are artifacts of our isolation procedure or sucrose gradient centrifugation cannot be answered at this time.

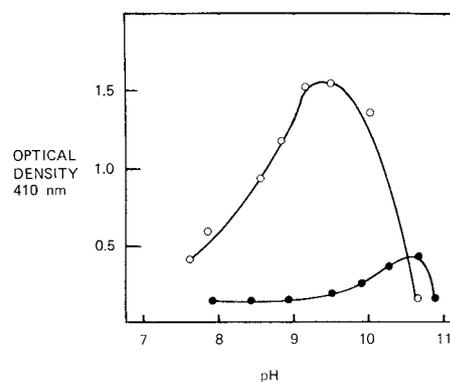


FIG. 6. Effect of pH on the activity of purified alkaline phosphatase from *Thermus aquaticus*. Purified alkaline phosphatase (0.003 $\mu\text{g}/\text{assay tube}$) was assayed in a reaction mixture containing 1 mM CaCl_2 and 1 mM *p*-nitrophenyl phosphate. The reactions were run at 75° for 10 min and corrections were made for the temperature coefficients of the buffers. \circ — \circ , Activity in 0.5 M Tris buffer; \bullet — \bullet , activity in 0.25 M glycine-NaOH buffer.

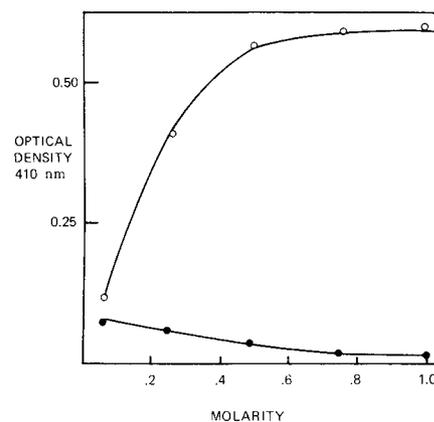


FIG. 7. Effect of the buffer concentration on the activity of the purified alkaline phosphatase. The enzyme (0.003 $\mu\text{g}/\text{assay tube}$) was assayed at 75° as described under "Experimental Procedures." In addition, a control was run at each Tris concentration without enzyme and the amount of nonenzymatic hydrolysis of the substrate caused by the increased Tris concentration was negligible in contrast to the amount of enzymatic hydrolysis caused by that increase. \circ — \circ , Activity in Tris buffer pH 9.0; \bullet — \bullet , activity in glycine-NaOH, pH 9.6.

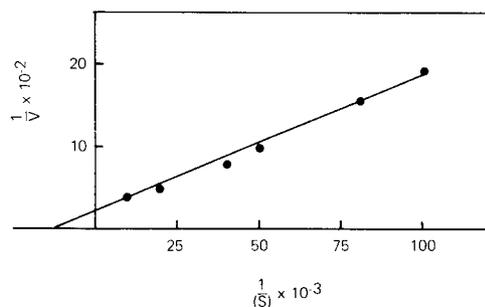


FIG. 8. Determination of the Michaelis constant (K_m) for alkaline phosphatase by the method of Lineweaver and Burk (22). The enzyme (0.002 $\mu\text{g}/\text{assay tube}$) was assayed with various concentrations of *p*-nitrophenyl phosphate at 75° in 0.5 M Tris buffer, pH 9. The S stands for the molarity of *p*-nitrophenyl phosphate and the V for micromoles of nitrophenol/min liberated at that given substrate concentration.

TABLE III

Substrate specificity of alkaline phosphatase from *Thermus aquaticus*

Purified alkaline phosphatase (0.02 μ g) was added to different reaction mixes containing 0.5 M Tris pH 9, 1 mM CaCl₂, and 10 mM substrate. The reaction was run at 75° for 10 min. The orthophosphate liberated was measured by the method of Fiske and SubbaRow (16).

Substrate ^a	Relative amount of orthophosphate liberated during enzymatic hydrolysis ^b
<i>p</i> -Nitrophenyl phosphate	1.00
α -Sodium glycerol phosphate	1.09
β -Sodium glycerol phosphate	0.89
Glucose 6-phosphate	0.80
Pyridoxal 5-phosphate	0.04
α -D-Glucose 1-phosphate	0.60
Adenosine triphosphate	0.98
Adenosine diphosphate	0.38
Pyrophosphate	0.00

^a Since some of the substrates were unstable, a control for each was run without enzyme at 75°.

^b The amount of orthophosphate liberated from *p*-nitrophenyl phosphate by the enzyme was used as a standard in comparing the relative amounts of orthophosphate liberated from the other substrates.

Two of the more interesting aspects of this enzyme are its broad temperature range and the stimulation of its catalytic activity by Tris buffer. Optimum activity was only 3 times higher than the activity seen at 20°. These data are in contrast to reports on other enzymes from *T. aquaticus* such as acetohydroxy acid synthetase (23), aldolase (3), and DNA polymerase¹ where these enzymes had catalytic activities at 20° of less than 5% of the activities seen at their respective temperature optima. The observed enhancement by high concentrations of Tris may be due to transphosphorylation between Tris and substrate, a situation which has been reported for the alkaline phosphatase from *E. coli* (24).

Another intriguing aspect of this study is that these thermophilic bacteria, living at a biological extreme, have maintained regulatory systems similar to those found in mesophilic bacteria. Like alkaline phosphatases from *E. coli* and many other bacterial sources, the thermophilic alkaline phosphatase is derepressible and the relative levels of derepression varied over a range of more than 1,000-fold depending upon the growth conditions. Sodium glycerol phosphate gave the best derepression and this was probably due to the slower availability of its phosphate for cellular use than that of the same molar amount of inorganic phosphate as indicated by the slower growth rate of *T. aquaticus* in limiting amounts of sodium glycerol phosphate as compared to the growth rate in the same molar amount of inorganic phosphate. Coupled with the report that the isoleucine-valine biosynthetic enzymes are under the control of multivalent repression and the acetohydroxy acid synthetase is feedback inhibited by valine (23), these data led to the conclusion that adaptation to life at elevated temperatures is possible without sacrificing control mechanisms similar to those in mesophiles such as *E. coli*. This conclusion contradicts concepts put forth by Brock (25) who suggests that thermophiles might have sacrificed efficiency and control mechanisms in order to be able to grow at a biological extreme. Furthermore, it is difficult to visualize *T. aquaticus* as a primordial life form as has often been suggested; it must be

¹ Unpublished data.

TABLE IV

Amino acid composition of alkaline phosphatase from *Thermus aquaticus*

Amino acid	Residues/51,000 \times g protein	Nearest integer/51,000 \times g
Aspartic	41.8	42
Glutamic	49.4	49
Proline	22.8	23
Glycine	43.4	43
Alanine	58.1	58
Valine	37.7	38
Isoleucine	14.8	15
Leucine	44.3	44
Tyrosine	19.2	19
Phenylalanine	19.7	20
Histidine	10.4	10
Lysine	20.4	20
Arginine	31.2	31
Methionine ^a	15.4	15
Threonine ^b	22.2	22
Serine ^b	23.8	24
Half-cystine ^a	2.2	2
Tryptophan ^c	9.7	10

^a Measured as methionine sulfone and cysteic acid, respectively.

^b Values extrapolated to zero time of hydrolysis.

^c Measured by the spectrophotometric method of Edelhoch (20).

seen as an advanced life form which has evolved along with bacteria such as *E. coli* because it has many control mechanisms which are generally thought to be advanced.

It should be pointed out that although the enzyme is stable for a relatively long period of time there is some variation in its specific activity and this may be due to anything from conformational changes to our experimental procedures.

The location of alkaline phosphatase is quite different in various microorganisms. For example, in *E. coli*, it is in their periplasmic space; in *Neurospora crassa*, intracellular; and in *M. sodonensis*, extracellular. The material used for purification of *T. aquaticus* alkaline phosphatase was isolated from the pellet after centrifugation, but a significant portion of enzymatic activity was also observed in the growth medium. Whether or not these two differently located alkaline phosphatases are the same is currently being studied in our laboratory.

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