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

Min-fung Yeh and John M. Treba

From the Department of Biological Sciences, University of Cincinnati, Cincinnati, Ohio 45221

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 41,000. 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 phosphorlated compounds as do many of the mesophilic alkaline phosphatases. The Michaelis constant ($K_m$) for the enzyme is $8.0 \times 10^{-4}$ 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-amyase (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* (1), *Bacillus licheniformis* (8), *Micrococcus 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 0.2% 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; CaSO$_4$·2H$_2$O, 60 mg; MgSO$_4$·7H$_2$O, 100 mg; NaCl, 8 mg; KNO$_3$, 103 mg; NaNO$_3$, 689 mg; ZnSO$_4$·5H$_2$O, 5 mg; H$_3$BO$_3$, 5 mg; CuSO$_4$·5H$_2$O, 16 mg; NaMoO$_4$·2H$_2$O, 0.25 mg; CoCl$_2$, 0.4 mg; FeCl$_3$, 0.28 mg; and MnSO$_4$·H$_2$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-kr magnetostriective ultrasonic oscillator, operated at 5.5 A for 30 s. The extracts were then spun in a Sorvall RC-2B centrifuge at 27,000 × $g$ 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
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 μmol 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 × 0.5 cm) contained 7% acrylamide, whereas the spacer gel (15 × 0.5 cm) contained 2% acrylamide. Electrophoresis, in a Canalo model 66 electrophoresis bath, was carried out at room temperature in 2.5 mm Tris/1.9 mm glycine buffer, pH 8.5, for 9.5 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 Tidecyl 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 (M₀ = 94,000), serum albumin (M₀ = 67,000), ovalbumin (M₀ = 43,000), and chymotrypsinogen (M₀ = 25,700).

**Sedimentation Coefficient**—Purified alkaline phosphatase (10 μg) and serum albumin (2.7 mg) were layered on a linear sucrose gradient of 6 to 25%. The gradient was made up of 0.02 M Tris buffer, pH 8.0, and 1 mm CaCl₂. Centrifugation was performed using a Spinco SW 50.1 rotor in a preparative Beckman ultracentrifuge at 38,000 rpm for 17 hours at 4°C. 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°C. 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).

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**—The pooled fractions were then dialyzed against 10 mM potassium phosphate, pH 8, containing 1 mm CaCl₂. 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°C without appreciable loss of activity for at least a period of 2 months.

**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 itself is not proof of homogeneity, although the results are consistent with the results of other methods of analysis and are in agreement with the electrophoretic data.

**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 eluted 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 (M₀ = 520,000).
TABLE II
Summary of purification procedure

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Total alkaline phosphatase activity*</th>
<th>Total protein</th>
<th>Yield of activity</th>
<th>Specific activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>248</td>
<td>213,000 mg</td>
<td>23,718 mg</td>
<td>78.4%</td>
<td>78.4 units/mg</td>
<td>3.5</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>500</td>
<td>60,000 mg</td>
<td>220 mg</td>
<td>28%</td>
<td>78.4 units/mg</td>
<td>3.5</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>200</td>
<td>61,000 mg</td>
<td>60 mg</td>
<td>28%</td>
<td>1,023 units/mg</td>
<td>13.0</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>35</td>
<td>39,700 mg</td>
<td>30.1 mg</td>
<td>19%</td>
<td>1,320 units/mg</td>
<td>16.9</td>
</tr>
</tbody>
</table>

*One unit of alkaline phosphatase equals the release of 1 μmol of nitrophenol/min at 75°C.

# Temperature Optimum
Since Thermus aquaticus is an extreme thermophile which can grow at temperatures up to 80°C, 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°C, 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°C. The activity was considerably higher in Tris buffer than in glycine/NaOH buffer (Fig. 6). The pH optimum was approximately 8.2. 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).

# Substrate Specificity
The substrate specificity was determined for p-nitrophenyl at 75°C. 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 spondensis (9), and Bacillus licheniformis (8).

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).

In summary, the purified enzyme from Thermus aquaticus exhibits broad temperature and pH optima, high substrate specificity, and a molecular weight of approximately 51,000. This enzyme represents a valuable tool for studying alkaline phosphatases in extreme thermophiles.
Alkaline Phosphatase from Thermus aquaticus

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 µg/assay tube) was incubated for 10 min at different temperatures. The reaction mixture contained 0.5 M Tris buffer, pH 9, 1 mM CaCl₂, 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 x 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 µg/assay tube) was assayed in a reaction mixture containing 1 mM CaCl₂ 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. - O- Activity in 0.5 M Tris buffer; - ●, 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 µg/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. - O-- Activity in Tris buffer pH 9.0; - ● -●, 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 µg/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.
primordial life form as has often been suggested; it must be thermophiles might have sacrificed efficiency and control to those in mesophiles such as E. coli. This conclusion further supports the idea that T. aquaticus alkaline phosphatase was isolated 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°C. These data are in contrast to reports on other enzymes from T. aquaticus 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.

Acknowledgments—The authors wish to thank Drs. Antony Mukkada, Bruce Umminger, Ralph Meyer, and Steve Keller for valuable discussions and for critically reading the manuscript. We would also like to thank Dr. James Freisheim for helping with the amino acid composition and Mr. David Stalker with the gel electrophoresis.

REFERENCES

Table I
Substrate specificity of alkaline phosphatase from Thermus aquaticus

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>Relative amount of orthophosphate liberated during enzymatic hydrolysis a</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Nitrophenyl phosphate</td>
<td>1.00</td>
</tr>
<tr>
<td>a-Sodium glycerol phosphate</td>
<td>1.09</td>
</tr>
<tr>
<td>b-Sodium glycerol phosphate</td>
<td>0.89</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>0.80</td>
</tr>
<tr>
<td>Pyridoxal 5-phosphate</td>
<td>0.04</td>
</tr>
<tr>
<td>a-D-Glucose 1-phosphate</td>
<td>0.60</td>
</tr>
<tr>
<td>Adenosine triphosphate</td>
<td>0.98</td>
</tr>
<tr>
<td>Adenosine diphosphate</td>
<td>0.38</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>0.00</td>
</tr>
</tbody>
</table>

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

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.

### Table IV
Amino acid composition of alkaline phosphatase from Thermus aquaticus

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues/50,000 g protein</th>
<th>Nearest integer/50,000 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic</td>
<td>41.8</td>
<td>42</td>
</tr>
<tr>
<td>Glutamic</td>
<td>49.4</td>
<td>49</td>
</tr>
<tr>
<td>Proline</td>
<td>22.8</td>
<td>23</td>
</tr>
<tr>
<td>Glycine</td>
<td>43.4</td>
<td>43</td>
</tr>
<tr>
<td>Alanine</td>
<td>58.1</td>
<td>58</td>
</tr>
<tr>
<td>Valine</td>
<td>37.7</td>
<td>38</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>14.8</td>
<td>15</td>
</tr>
<tr>
<td>Leucine</td>
<td>44.3</td>
<td>44</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>19.2</td>
<td>19</td>
</tr>
<tr>
<td>Phenylalanine</td>
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<td>20</td>
</tr>
<tr>
<td>Histidine</td>
<td>10.4</td>
<td>10</td>
</tr>
<tr>
<td>Lysine</td>
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<tr>
<td>Arginine</td>
<td>31.2</td>
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<tr>
<td>Methionine</td>
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<tr>
<td>Threonine</td>
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<td>22</td>
</tr>
<tr>
<td>Serine</td>
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<td>24</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>2.2</td>
<td>2</td>
</tr>
<tr>
<td>'Tryptophan'</td>
<td>9.7</td>
<td>10</td>
</tr>
</tbody>
</table>

* Measured as methionine sulfone and cysteic acid, respectively.

Values extrapolated to zero time of hydrolysis.

* Measured by the spectrophotometric method of Edelhoch (20).
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