Purification and Properties of Bovine Synovial Fluid Alkaline Phosphatase*

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SUMMARY
Alkaline phosphatase from bovine synovial fluid was purified 2300-fold. A molecular weight of 72,300 was determined from sucrose density gradient studies. The following monoesters were hydrolyzed by the enzyme: β-glycerophosphate, galactosaminic 6-phosphate, glucosaminic 6-phosphate, glucose 6-phosphate, o-phospho-l-serine, o-carboxyphenyl phosphate, phenyl phosphate, and p-nitrophenyl phosphate. Kinetic studies of the enzyme were made with p-nitrophenyl phosphate as substrate. Adenosine triphosphate and pyrophosphate were not hydrolyzed by the enzyme.

Activation of the enzyme was observed with Sr2+, Ca2+, and Mg2+ ions. Cyanide and fluoride, as well as ethylenediaminetetraacetate, inhibited the enzyme activated by Mg2+ while the chelating agent, 1,2-bis-(2-dicarboxymethylaminoethoxy)ethane (EGTA), inhibited the enzyme activated by Ca2+. Diisopropylfluorophosphate and p-chloromercuribenzoate were virtually without effect on the activity.

The pH optimum increased with increasing substrate concentration. The pH profiles were obtained for pK, log V max, and log V max/K m. These data show the existence of two groups at the active site having pK values of 8.6 and 9.6.

Non-specific alkaline phosphatases (EC 3.1.3.1) are present in a variety of tissues, among which are the kidneys, intestine, and connective tissues (1–4). The latter has been implicated in the immediate process of ossification (5) yet the validity of this proposal is still open to question. Before meaningful progress can be made toward understanding the function of alkaline phosphatase in connective tissues, it is essential to study the properties of the purified enzyme.

Because of its comparatively high concentration in alkaline phosphatase, synovial fluid of yearling cattle was chosen as the source of the enzyme. The specific activity of the enzyme in synovial fluid may be as much as 100 times that of the serum.

A method for purifying alkaline phosphatase from bovine synovial fluid is described in this report, as are the effects of pH, divalent cations, and inhibitors on enzymatic activity. The substrate specificity of the enzyme is also reported.

EXPERIMENTAL PROCEDURE
Sampling and Treatment of Synovial Fluid—Synovial fluid was obtained from the astragalotibial joints of yearling heifers or steers. Only those samples free of blood were pooled and used for these studies. The fluid was centrifuged at 1000 × g for 45 min to remove cellular debris. For every 10 ml of clear, viscus synovial fluid, 2 mg of hyaluronidase were added and the resulting solution was kept at 5° for 48 hours or until the viscosity dropped to a minimal value. The treated synovial fluid was used immediately or kept frozen until needed. Storage for periods from 6 to 8 months did not cause any apparent loss in enzymatic activity.

Assay Procedures—Alkaline phosphatase activity was measured according to a modified version of the method of Lowry et al. (8). Samples from various stages of the isolation procedure were assayed with the use of p-nitrophenyl phosphate as substrate. The assay mixture contained 0.10 M 2-amino-2-methyl-1-propanol-HCl buffer, pH 10.1 (at 25°), 0.0025 M MgCl2, and 0.0036 M substrate in a total volume of 2 ml. After the mixture had been incubated at 37° for 30 min, the reaction was stopped by the addition of 2 ml of 0.5 N HCl. The absorbance was measured at 405 nm and the specific activity was calculated as units per mg of protein.

The authors wish to acknowledge the cooperation of the Standard Beef Company, Detroit, for the synovial fluid.

The abbreviations used are: TEAE, triethylaminoethyl; EGTA, 1,2-bis(2-dicarboxymethylaminoethoxy)ethane, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid.

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stopped by adding 10 ml of 0.02 N NaOH and the resulting yellow color was measured at 410 μm. One unit of activity was defined as that amount of enzyme required to liberate 0.05 μmole of p-nitrophenol in 30 min.

The conditions for the kinetic assays were as described above except that the final volume was increased to 3 ml. When p-nitrophenyl phosphate was used as substrate, the release of p-nitrophenol was measured at 410 μm in a temperature-regulated, recording spectrophotometer. For o-carboxyphenyl phosphate, the release of salicylic acid was measured at 300 μm (9), whereas phenol formation resulting from the hydrolysis of phenyl phosphate was measured at 287 μm (10).

All other substrates were studied by analyzing for inorganic phosphate with the N-phenyl-p-phenylenediamine reagent (11). Assays for phosphate were performed after 0, 5, and 10 min of incubation to ensure linearity of the reaction with time.

The protein concentration was determined from the optical density at 280 μm, as suggested by Warburg and Christian (12).

**Preparation of Antiglial Fluid Antiserum**—Equal quantities (0.50 ml) of depolymerized synovial fluid and Freund’s adjuvant were mixed and injected subcutaneously into New Zealand albino rabbits. Injections were continued weekly for 1 month. After this time interval, the rabbit serum was checked by immunoelectrophoresis. Booster injections were then given at 2-week intervals.

**Materials**—Sigma “104” phosphatase substrate (p-nitrophenyl phosphate), β-glucose 6-phosphate, and α- and β-naphthyl phosphates were purchased from Sigma. Monophenyl phosphate and o-carboxyphenyl phosphate were obtained from Mann. β-Glycerophosphoric acid was obtained from Eastman, and o-phospho-L-serine was provided by Dr. F. C. Neuhaus of the Department of Chemistry, Northwestern University. Glucose 6-phosphate and galactosamine 6-phosphate were obtained from Dr. Saul Roseman of the Rackham Arthritis and Research Unit, Ann Arbor, Michigan.

Hyaluronidase, 300 U.S.P. units per mg, was purchased from Nutritional Biochemicals.

DEAE-cellulose (Cellex-D) and TEAE-cellulose (Cellex-T), high capacity ion exchange resins, were obtained from Calbiochem, as was the diazotized o-chlorotoluidine which was used for the isolation of alkaline phosphatase from agar gels.

The 2-amino-2-methyl-1-propanol (Sigma) was distilled under reduced pressure prior to use.

The collodion bags and glass apparatus used for pressure dialysis were obtained from Schleicher and Schuell, Keene, New Hampshire.

**RESULTS**

**Purification of Alkaline Phosphatase**—A typical preparation of alkaline phosphatase is described below; the results are summarized in Table I. The entire procedure was performed at 5°C.

Hyaluronidase-treated synovial fluid (1300 ml) was added to an equal volume of cold distilled water, and 0.351 g of (NH₄)₂SO₄ per ml were added slowly to bring the solution to 55% of saturation (13). The mixture was allowed to equilibrate for 1 to 2 hours prior to centrifugation at 12,000 × g for 15 min. The supernatant solution was brought to 75% of saturation by adding 0.141 g of (NH₄)₂SO₄ per ml of supernatant. After stirring for 1 hour, the mixture was centrifuged at 12,000 × g; the supernatant solution was decanted and discarded. The enzyme-rich precipitate, P₁, was dissolved in a minimum quantity of 0.05 M Tris-HCl buffer (pH 7.6) containing 0.1 M NaCl. The yellow solution was dialyzed overnight against three changes of 4 liters each of the buffer. The solution of enzyme was subsequently dialyzed against three changes of 4 liters each of 0.05 M Tris-HCl (pH 8.8) containing 0.001 M MgCl₂.

The dialyzed solution of enzyme, 122 ml containing 19 mg of protein per ml, was applied to a DEAE-cellulose column (4.2 cm × 8 cm) previously equilibrated with 0.05 M Tris-HCl buffer (pH 8.8) containing 0.001 M MgCl₂. The column was eluted with a linear MgCl₂ gradient formed from 1 liter of the buffer used for the equilibration and 1 liter of 0.05 M Tris-HCl (pH 8.8) containing 0.1 M MgCl₂. Fractions of 3 ml were collected. The elution pattern showed a single peak of phosphatase activity, which preceded the first protein peak slightly. The enzyme-rich fractions, E₁, were pooled and carried directly through the rest of the procedure. Fractions of low specific activity were combined and passed through a fresh DEAE-cellulose column under identical conditions. In general, the yield of purified enzyme obtained from each of the ion exchange steps was increased by recycling the fractions of low activity.

Fraction E₁ was dialyzed against three changes of 4 liters each of 0.05 M Tris-HCl (pH 8.3) containing 0.001 M MgCl₂. The volume of enzyme solution was then reduced by pressure dialysis in collodion bags. The concentrated enzyme solution was applied to a TEAE-cellulose column (3 × 33 cm) previously equilibrated with 0.05 M Tris-HCl (pH 8.3) containing 0.001 M MgCl₂. The column was eluted with a linear MgCl₂ gradient formed with 500 ml of initial buffer and 500 ml of the same buffer, which was 0.1 M in MgCl₂. A single peak of phosphatase activity, partially overlapping a slower protein peak, was obtained. The fractions which were rich in enzyme E₂ were pooled for subsequent purification. The combined fractions of lower activity were recycled.

Fraction E₂ was dialyzed against three changes of 4 liters each of 0.05 M Tris-HCl buffer (pH 8.3) containing 0.001 M MgCl₂. The dialyzed solution was applied to a second TEAE-cellulose column (1.5 × 22 cm) equilibrated with the same concentration of Tris buffer as used for dialysis. The column was eluted with a linear NaCl gradient formed by 300 ml of the buffer used for equilibration and 300 ml of the same buffer containing 0.2 M NaCl.

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Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total units</th>
<th>Specific activity units/mg protein</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synovial fluid: H₂O</td>
<td>93,000</td>
<td>7</td>
<td>100 %</td>
<td>fold</td>
</tr>
<tr>
<td>P₁: precipitate at 55-75% (NH₄)₂SO₄ saturation</td>
<td>91,500</td>
<td>38</td>
<td>98.5</td>
<td>5.4</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography of P₁: E₁</td>
<td>48,510</td>
<td>189</td>
<td>59.2</td>
<td>27.0</td>
</tr>
<tr>
<td>TEAE-cellulose chromatography of E₁ with Mg⁺⁺ gradient: E₂</td>
<td>20,150</td>
<td>545</td>
<td>21.7</td>
<td>78.0</td>
</tr>
<tr>
<td>TEAE-cellulose chromatography of E₂ with Na⁺ gradient: E₃</td>
<td>11,070</td>
<td>4,200</td>
<td>11.9</td>
<td>600.0</td>
</tr>
<tr>
<td>Sucrose density gradient centrifugation of E₄: C₁</td>
<td>7,200</td>
<td>16,000</td>
<td>7.8</td>
<td>2,286</td>
</tr>
</tbody>
</table>
The fractions with maximal specific activity, $E_4$, were again pooled, and pressure-dialyzed in collodion bags against 0.08 M Tris-HCl buffer (pH 8.3) containing 0.001 M MgCl$_2$. Fractions of lower specific activity were saved for recycling. The specific activity of Fraction $E_4$ was often as high as 4500, depending on the care exercised with the final chromatographic step.

The material at this stage of purification, $E_4$, yielded a single symmetrical peak upon sedimentation velocity analysis (Fig. 1). This preparation was also subjected to centrifugation in an Yphantis-Waugh cell until the protein peak was concentrated in the lower chamber. Analyses for alkaline phosphatase showed that all of the activity was located in the lower chamber. The enzyme, therefore, appeared to be associated with the protein forming the boundary. The possibility that a heavier material might in reality have been responsible for the activity could not be ruled out by these experiments.

The preparation of enzyme was free of pyrophosphatase (14) and diesterase activities (15). It could not be resolved further by molecular sieving on columns of Sephadex G-75 to G-200 or columns of acrylamide prepared according to Hjerten and Mosbach (16). The specific activity was not increased by adsorption on calcium apatite or Mg(OH)$_2$ gels or by electrophoresis on starch block. Agar gel electrophoresis (17) showed that the area which stained for protein also gave a positive phosphatase reaction when assayed with $\alpha$- or $\beta$-naphthyl phosphate in the presence of diazotized o-chlorotoluidine.

Immunoelectrophoresis (17) with the use of antilidene synovial fluid rabbit antiserum showed the presence of three to five bands of antigenic material in the purified preparation (Fig. 1), thereby introducing a serious doubt as to its homogeneity. As a further test, the preparation was centrifuged in a sucrose density gradient according to the method of Martin and Ames (18). Fig. 2 shows the distribution of protein and phosphatase activity. Tubes 12 through 18 were pooled to give Fraction $C_1$, as were tubes 19 through 22 and 23 through 28. Fraction $C_1$, with a specific activity of 16,000 (Table I), was in turn subjected to immunoelectrophoresis and was found to be devoid of contaminating material. Therefore, sucrose gradient centrifugation provided a method for obtaining enzyme of maximal purity from a system which had proved to be refractory to a number of conventional procedures.

A molecular weight of 72,500 was calculated for the enzyme from the results of the density gradient experiments. Crystalline bovine serum albumin was used as the reference protein. This value is in good agreement with results for other alkaline phosphatases (19).

Effect of pH on Velocity and $V_{max}$—Investigations with alkaline phosphatase from various sources (20, 21) have demonstrated a dependence of pH optimum on substrate concentration. This dependence is illustrated in Fig. 3 for the purified enzyme from synovial fluid with p-nitrophenyl phosphate as substrate. Analogous results were found for the unfractionated material. The curves pass through maxima which shift to more basic pH values with increasing substrate concentration. The shift in optimum is not restricted to this particular substrate, for similar observations were reported by Morton for the intestinal enzyme with phenyl phosphate (20) and by Motzok for plasma enzymes with $\beta$-glycerophosphate (21).

The logarithms of maximal velocity, $V_{max}$, and of the velocity of hydrolysis at high concentration of p-nitrophenyl phosphate, 5 mM, were found to reach maximum values at a pH of 9.5 (Fig. 4a).

Effect of pH on $pK_a$—Michaelis constants were calculated from a series of Lineweaver-Burk plots obtained at various pH values with the use of p-nitrophenyl phosphate. Fig. 4b relates...
the logarithm of the reciprocal of the $K_m$ ($pK_m$) to the pH. The important feature of the pH profile is the diminution of the $pK_m$ in the pH range where the enzyme exhibits maximal activity. This indicates a pH dependence of $K_m$ when $V_{max}$ or the velocity ($V$) at large substrate concentration is independent of pH. Similar results were obtained by Morton (20) with the intestinal enzyme when phenyl phosphate was used as substrate and by Jacobson (22) when $\beta$ glycerophosphate was used for the kidney enzyme.

The inflection point on the $pK_m$-pH diagram (Fig. 4b) is usually interpreted as corresponding to the ionization of a functional group on the enzyme involved in the formation of a rate-determining complex (23). This interpretation is applicable if the enzyme forms a single enzyme-substrate intermediate. If, on the other hand, a number of enzyme-substrate complexes are formed—and there is reason to believe this is true for alkaline phosphatases (24)—the experimentally derived value for $K_m$ then represents a complex kinetic function (25) and the change in $K_m$ with pH may instead reflect a change in the rate controlling step. The data shown in Fig. 4b actually represent the variation of the apparent $K_m$ with pH.

**Effect of pH on log $V_{max}/K_m$**—Peller and Alberty (26) and Krupka and Laidler (27) have developed a method for the determination of the $pK$ values of functional groups of the free enzyme which are essential for enzymatic activity. These values are readily determined from the inflection points of the pH profiles of log $V_{max}/K_m$. The $pK$ values are observed from Fig. 4c to be 8.6 and 9.6. These two values are separated by only 1 pH unit and, therefore, may be in error since a separation of several pH units is necessary for unambiguous results.

**Activation by Divalent Metal Cations**—Alkaline phosphatase from synovial fluid is markedly activated by such divalent cations as $Mg^{2+}$, $Ca^{2+}$, and $Sr^{2+}$. The effects of the metal ions on the rate of phosphatase activity are shown in Fig. 5. Since the maximal velocities attained with all cations except zinc were identical, it was convenient to compare the results obtained at various ion concentrations as the percentage of the maximal activity. In the absence of metal ions, the rate fell virtually to zero. The concentration of divalent ion required to reach maximal activity decreases as one progresses down the periodic table, i.e. Mg, Ca, Sr. Zinc, on the other hand, acts as an activator at very low concentrations ($10^{-5}$ M) and inhibits the enzyme in the range where the other cations are optimally active (Fig. 5).

**Inhibition Studies**—Complete inhibition of the alkaline phosphatase activated by $Mg^{2+}$ was observed after the addition of at least an equimolar (0.0025 M) quantity of EDTA to an assay mixture which was 0.0025 M in magnesium (Fig. 6). It is also apparent from Fig. 6 that cyanide inhibited at levels equimolar

![Fig. 3. Relationship between pH and the rate of hydrolysis of different concentrations of p-nitrophenyl phosphate. Amount of p-nitrophenol liberated was measured at the indicated pH (37°). The curves are for the following substrate concentrations (millimolar): ■, 0.05; □, 0.1; O, 0.5; ●, 5.0. The specific activity of the enzyme was 4500.](image1)

![Fig. 4. The pH profiles of log $V$, $pK_m$, and log $V_{max}/K_m$ for p-nitrophenyl phosphate. a, the upper curve illustrates the pH dependence of log $V_{max}$ and the lower curve that of log $V$ measured at 5 mM substrate; b, the effect of pH on $pK_m$; c, the variation of log $V_{max}/K_m$ with pH. O, log $V_{max}$; ●, log $V$.](image2)

![Fig. 5. Effect of activator concentration on the rate of hydrolysis of p-nitrophenyl phosphate. The negative logarithm of the molar concentration of activator in the incubation mixture is shown in the diagram. The velocity measurements were made as indicated under "Experimental Procedure" with 3.5 units of enzyme of specific activity of 4500 in the cuvette with the exception that the standard amount of MgCl$_2$ was omitted. The enzyme was assayed instead in the presence of various final concentrations of ZnCl$_2$ (●), CaCl$_2$ (□), Sr(NO$_3$)$_2$ (■), and MgCl$_2$ (■).](image3)
TABLE II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Nitrophenyl phosphate</td>
<td>$2.45 \times 10^{-4}$</td>
</tr>
<tr>
<td>Monophenyl phosphate</td>
<td>$3.56 \times 10^{-4}$</td>
</tr>
<tr>
<td>o-Carboxyphenyl phosphate</td>
<td>$8.18 \times 10^{-3}$</td>
</tr>
<tr>
<td>p-Glucose 6-phosphate</td>
<td>$5.08 \times 10^{-4}$</td>
</tr>
<tr>
<td>$\beta$-Glycerophosphoric acid</td>
<td>$4.06 \times 10^{-3}$</td>
</tr>
<tr>
<td>o-Phospho-L-serine</td>
<td>$3.24 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

The metal-activated enzyme displayed a broad specificity for the organic moiety of the substrate to the enzyme as in phosphoserine phosphatase (33), or, as suggested by Boyer and Harrison (34), they may serve to neutralize the negative charges on the phosphate moiety so that nucleophilic attack of the phosphorus atom by hydroxyl ions can take place. This suggestion seems plausible in the light of studies showing the incorporation of $^{38}O$ from labeled water into the phosphate group (35). The possibility that magnesium ions also have a function similar to that of calcium in amylase (36), namely, to impart a structural rigidity essential for catalytic activity, cannot as yet be rejected. Experiments to clarify the function of metal activators in the alkaline phosphatase of rabbit small intestine lend strong support to this idea.

As expected, metal-binding agents such as cyanide, fluoride, EDTA, and EGTA are effective inhibitors. Specific group reagents, on the other hand, such as p-chloromercuribenzoate, had no effect on the enzyme, thus eliminating accessible sulfhydryl groups as essential to the active site. At high concentrations, diisopropyl fluorophosphate inhibited to the extent of only 12%, perhaps reflecting the inability of the enzyme to react with diesters or the absence of unique structural requirements necessary for reaction with this reagent.

The pH optima showed a dependence on substrate concentration. An explanation for this phenomenon must take into account changes in the ionization of groups at the active site of the enzyme resulting from attachment of the substrate. An additional important feature of the reaction of alkaline phosphatase with its substrates is the formation of a phosphoryl intermediate.
(24) during the course of the reaction. The sequence of events is believed to proceed according to the following equations (24).

\[ E + \text{ROH} \rightleftharpoons E \cdot \text{ROH} \]
\[ E \cdot \text{ROH} \rightleftharpoons E \cdot \text{P} + \text{ROH} \]
\[ E \cdot \text{P} + \text{H}_2\text{O} \rightleftharpoons E + \text{Pi} \]

E designates the enzyme, ROH the alcohol, P the phosphoryl group, ROP the phosphate ester, and E-P the phosphoryl enzyme.

Krupka and Laidler (27) have derived a mathematical expression for pH optima of enzymes which form a number of intermediates.substrate concentration is a variable in the equation and, therefore, influences the value observed for the pH optimum. Complete evaluation of the equation, however, must await a knowledge of the ionization constants (or pK values) of all the groups involved in the reaction and the velocity constants for the reactions indicated above.

The current investigations permit an evaluation of the pK value of two groups at the active site. These were found to be 8.6 and 9.6, respectively, from the inflection points of the pH profile of log \( V_{\text{max}}/K_m \). Although these two values have not been assigned to specific groups, the phenox component of tyrosine and the \( \epsilon \)-NH2 of lysine have been suggested (20) for the structure with the higher pK value. Additional support is needed from group-specific reagents. Both of the pK values are, however, well below that for the serine hydroxyl (pK = 13.5) (37), which has been demonstrated to be phosphorylated in phosphatases isolated from intestine and from E. coli. It is conceivable that at least three groups are required at the active site, as shown recently by Bender and Köédy (38) for chymotrypsin. Only two groups, however, were determined from the pH profile of log \( V_{\text{max}}/K_m \) for alkaline phosphatase.

Morton has interpreted the inflection point of the pH-pK curve as the pK of a group involved in the formation of the enzyme-substrate complex, while the more recent work of Bender et al. (25) shows that the inflection may represent a change in the rate-limiting step. To distinguish between these two possibilities, a number of substrates exhibiting different rate-limiting steps need to be studied. The inflection point of a pH of 9.2 for intestinal phosphatase with phenyl phosphate as substrate. Bender et al. (25) have shown that this type of curve is the pK of a group involved in the formation of the enzyme-substrate complex, while the more recent work of Bender and KBzdy (38) for chymotrypsin. Only two groups, however, were determined from the pH profile of log \( V_{\text{max}}/K_m \) for alkaline phosphatase.

The pH profile of log \( V_{\text{max}} \) rises to a maximum and does not decline. Similar results were obtained by Wilson, Bayan, and Cyr (39) with the enzyme from E. coli and \( p \)-nitrophenyl phosphate as substrate. Bender et al. (25) have shown that this type of curve is formed when deacylation, or, in this case, dephosphorylation, is the rate-limiting step of the reaction at the pH values at which the enzyme is maximally active. Wilson et al. (39) demonstrated kinetically that this is indeed the case. This point needs to be firmly established now for the mammalian enzyme with the use of \( p \)-nitrophenyl phosphate as substrate. Measurement of the various rate constants for the reactions outlined above, as well as additional information regarding the structure of all essential groups at the active site, still needs to be completed before a clear picture of the mechanism of the enzyme may be advanced.

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REFERENCES

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