Purification and Properties of Bovine Synovial Fluid Alkaline Phosphatase

(Rceived for publication, June 1, 1965)

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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, galactosamine 6-phosphate, glucosamine 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 pH, divalent cations, and inhibitors on enzymatic activity. These data show the existence of two groups at the active site having pK values of 8.6 and 9.6.

Nonspecific 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). In the latter, the enzyme 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. The proteins in synovial fluid are derived in part from serum by virtue of a certain degree of capillary permeability. In addition, some are contributed by the surrounding cells (6). Preliminary electrophoretic studies showed a difference in mobilities between the alkaline phosphatases of synovial fluid and serum (7). This would indicate that the synovial fluid enzyme is derived directly from the synovial cells and not from 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 15 min to remove cellular debris. For every 10 ml of clear, viscous 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. Thawed samples of synovial fluid occasionally contained a white, flocculent, insoluble material which was removed by centrifugation. The precipitate was shown not to be bacterial contamination.

Preparation of Ion Exchange Derivatives—Prewashed DEAE- or TEAE-cellulose ion exchange derivatives were suspended in water and titrated to a predetermined pH with 0.5 N HCl. This step was repeated until the required pH was maintained. The cellulose was equilibrated against 5 volumes of starting buffer and packed into columns of the appropriate size.

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.

1 The authors wish to acknowledge the cooperation of the Standard Beef Company, Detroit, for the synovial fluid.
2 We thank Dr. Richard Berk, Department of Microbiology, Wayne State University, for establishing this point.
3 The abbreviations used are: TEAE, triethylaminoethyl; EGTA, 1,2-bis(2-dicarboxymethylaminoethoxy)ethane, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid.
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 Antisynovial 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), D-glucose 6-phosphate, and α- and β-naphthyl phosphates were purchased from Sigma. Monophenyl phosphate and o-carboxyphenyl phosphate were obtained from Mann. α-Glycerophosphoric acid was obtained from Eastman, and galactosamine 6-phosphate were obtained from Dr. Saul Roseman of the Rackham Arthritis and Research Unit, Ann Arbor, Michigan. Glu-cosamine 6-phosphate and galactosamine 6-phosphate were purchased from Sigma. Monophenyl phosphate with the N-phenyl-p-phenylenediamine reagent (11).

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 0.03 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 × 48 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 obtainable 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 0.03 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.

### Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total units</th>
<th>Specific activity</th>
<th>Yield (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synovial fluid: H₂O</td>
<td>98,000</td>
<td>7</td>
<td>100</td>
<td>1</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>93.2</td>
<td>97.0</td>
</tr>
<tr>
<td>TEAE-cellulose chromatography of E₁ with Mg²⁺ 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 E₃ on 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, $E_5$, were again pooled, and pressure-dialyzed in collodion bags against 0.03 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 $E_5$ was often as high as 4500, depending on the care exercised with the final chromatographic step.

The material at this stage of purification, $E_5$, 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 antihumal synovial fluid rabbit antisera 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). The sucrose solutions were prepared in 0.05 M Tris-HCl, pH 8.3, containing 0.001 M MgCl$_2$. The tubes were centrifuged in an SW 39 rotor for 16 hours at 4°C in a Spinco model L centrifuge at 50,000 rpm. Nine-drop fractions were collected, diluted with 0.20 ml of buffer, and assayed.

**FIG. 1.** Ultracentrifugal pattern and immunoelectropherogram of Fraction $E_5$. $A$, schlieren pattern obtained after 32 min at 52,040 rpm and at a phase plate angle of 55$^\circ$ in a synthetic boundary cell; $B$, immunoelectropherogram of $E_5$ in the upper well, and hyaluronidase-treated synovial fluid in the lower well. Antisyovial fluid serum was used in the trough. The anode is to the right of the origin.
the logarithm of the reciprocal of the $K_m$ ($\mu K_m$) to the pH. The important feature of the pH profile is the diminution of the $pK$ in the pH range where the enzyme exhibits maximal activity. This indicates a pH dependence of $K_m$ when $V_{\text{max}}$ 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.

**Fig. 4.** The pH profiles of log $V$, $pK_m$, and log $V_{\text{max}}/K_m$ for p-nitrophenyl phosphate. a, the upper curve illustrates the pH dependence of log $V_{\text{max}}$ 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 $V_{\text{max}}/K_m$ with pH. $\bigcirc$, log $V_{\text{max}}$; $\bigcirc$, log $V$.  

**Fig. 5.** Effect of activator concentration on the rate of hydrolysis of p-nitrophenyl phosphate. The negative logarithm of the molar concentration of salts 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$_2$ (□).
to the magnesium concentration while partial inhibition occurred
at a very low concentration. In contrast, fluoride ions were
required in high concentration, greater than 0.10 M, to produce
90% inhibition (Fig. 6). EGTA, which binds calcium specifi-
cally, was found to inhibit the Ca\(^{2+}\) activated enzyme.

Inubcation of the enzyme with 1.5 \times 10^{-4} M p-chloromercuri-
benzoate for 10 to 30 min at both 25° and 37° resulted in no loss of
activity. The p-chloromercuribenzoate incubations were
performed at pH 7.6 and 10.1.

Disopropyl fluorophosphate, 0.01 M, yielded only a 12% in-
hibition of the enzyme after 15 min of incubation at 37°, pH
7.6 and 10.1. A slight inhibition by relatively high concentra-
tions of disopropyl fluorophosphate was also observed by Webb
for crude kidney phosphatases (28).

Substrate Specificity—All of the phosphomonoesters tested as
substrates were hydrolyzed by the enzyme. The \(K_m\) values
were determined, when possible, from Lineweaver-Burk plots
and the results are presented in Table II. For galactosamine
6-phosphate and glucosamine 6-phosphate, the amounts avail-
able were insufficient for determinations of \(K_m\); therefore, the
rate of reaction relative to that of \(\beta\)-glycerophosphate at a final
substrate concentration of 0.004 M was determined. No sig-
nificant difference in the rate of release of inorganic phosphate
was detected.

**Table II**

<table>
<thead>
<tr>
<th>Compound</th>
<th>(K_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>(\alpha)-Carboxyphenyl phosphate</td>
<td>8.18 \times 10^{-3}</td>
</tr>
<tr>
<td>(\beta)-Glycerophosphoric acid</td>
<td>5.08 \times 10^{-4}</td>
</tr>
<tr>
<td>(\delta)-Gluco-6-phosphate</td>
<td>4.06 \times 10^{-3}</td>
</tr>
<tr>
<td>(\delta)-phospho-6-serine</td>
<td>3.24 \times 10^{-3}</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Despite the apparent homogeneity of the enzyme preparation
as revealed by sedimentation velocity analysis and the inability
to increase the specific activity by a number of fractionation
techniques, the preparation was shown to be heterogeneous
when examined by immunoelectrophoresis and sucrose density
centrifugation. These results emphasize the necessity of examin-
ing purified preparations of protein by several criteria.

Like other alkaline phosphatases, the synovial fluid enzyme is
a metal activated enzyme. Although it is recognized that the
known alkaline phosphomonoesters require the presence of
divalent cations for activity, there is no experimental evidence
establishing the function of the metal with the exception of the
zinc requirement for dimerization of the subunits of the en-
zyme from *Escherichia coli* (29). Engstrom showed that the
addition of magnesium ions to purified intestinal phosphatase,
which contains firmly bound zinc atoms, has no effect on phos-
phate binding, yet EDTA, hydrogen sulfide, and 1,10-phenan-
throne strongly inhibit phosphate incorporation (30). These
observations, coupled with the known requirement for diver-
ant cations for activity, indicate a dichotomy of function. The
two functional classifications refer to (a) the structure of the active
center (30) or of the enzyme itself (31), which requires indigenous
zinc atoms, and (b) the hydrolytic process, which is dependent on
added magnesium ions. The recent investigations of Clark and
Porteous (32) pertaining to metal ion activation of the alkaline
phosphatase of rabbit small intestine lend strong support to this
idea.

The magnesium ions may be required for attachment of 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 phos-
phate 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 \(^{3}O\) from la-
labeled water into the phosphate group (33). 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. Ex-
periments to clarify the function of metal activators in the alkaline
phosphatase of synovial fluid are in progress.

As expected, metal-binding agents such as cyanide, fluoride,
EDTA, and EGTA are effective inhibitors. Specific group re-
agents, 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,
disopropyl fluorophosphate inhibited to the extent of only 12%,
perhaps reflecting the inability of the enzyme to react with di-
esters or the absence of unique structural requirements neces-
sary for reaction with this reagent.

The metal-activated enzyme displayed a broad specificity for
substrates and would, therefore, be classified as one of the non-
specific phosphomonoesterases. Phosphate esters of aromatic
compounds, sugars, glycerol, and serine were hydrolyzed. Some
differences in \(K_m\) values were noted.

The pH optima showed a dependence on substrate concentra-
tion. An explanation for this phenomenon must take into ac-
count changes in the ionization of groups at the active site of the
enzyme resulting from attachment of the substrate. An addi-
tional important feature of the reaction of alkaline phosphatase
with its substrates is the formation of a phosphoryl intermediate

![Fig. 6. Effect of inhibitor concentration on the rate of magne-
sium-activated hydrolysis of \(p\)-nitrophenyl phosphate. The
molar concentration of cyanide (○), EDTA (●), and fluoride
(■) in the reaction mixture, which contained approximately 3.5
units of enzyme, Fraction E, is expressed in terms of the negative
logarithm. Other details are described in the text.](http://www.jbc.org/content/252/2/419.
fig.6)
The pH profile of log $V_{\max}/K_m$ 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 pH 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 pH profile of a 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 Keddy (38) for chymotrypsin. Only two groups, however, were determined from the pH profile of log $V_{\max}/K_m$ for alkaline phosphatase.

Morton has interpreted the pH-pK curve of the enzyme-substrate complex, while the more recent work of Bender et al. (25) shows that the pH 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 pH profile of a 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 Keddy (38) for chymotrypsin. Only two groups, however, were determined from the pH profile of log $V_{\max}/K_m$ for alkaline phosphatase.

The pH profile of log $V_{\max}$ 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 n-pitrophenyl phosphate as substrate. Bender et al. (25) have shown that this type of curve is found 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 n-pitrophenyl 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.

Acknowledgments—The authors would like to thank Drs. V. Massey and J. Schaefer of the University of Michigan for their stimulating discussion, which was very helpful in the preparation of this manuscript. We are also grateful to Dr. Charles Hamison of the Department of Physiology, Wayne State University, for his help in the ultracentrifugal studies. The technical assistance of Mr. Ralph Choiniere is acknowledged.

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