Purification and Partial Characterization of Alkaline Phosphatase of Matrix Vesicles from Fetal Bovine Epiphyseal Cartilage

PURIFICATION BY MONOCLONAL ANTIBODY AFFINITY CHROMATOGRAPHY*

(Received for publication, February 9, 1984, and in revised form July 27, 1984)

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Alkaline phosphatase of matrix vesicles isolated from fetal bovine epiphyseal cartilage was purified to apparent homogeneity using monoclonal antibody affinity chromatography. The enzyme from the butanol extract of matrix vesicles bound specifically to the immobilized antibody-Sepharose in the presence of 2% Tween 20 whereas the major portion of nonspecific protein was removed by this single step. Of various agents tested, 0.6 M 2-amino-2-methyl-1-propanol, pH 10.2, was the most effective in eluting 80–100% of the enzyme initially applied. Both Tween 20 and 2-amino-2-methyl-1-propanol associated with the eluted enzyme were effectively removed by the sequential application of DEAE-cellulose and Sepharose CL-6B chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the enzyme preparation treated with sodium dodecyl sulfate and mercaptoethanol showed the presence of a dominant band (using silver staining) corresponding to a molecular weight of 81,000. This molecular weight was nearer reported values for rat liver (Ohkubo, A., Langerman, N., and Kaplan, M. M. (1974) J. Biol Chem. 249, 7174–7180) and porcine kidney (Cathala, G., Brunel, C., Chappet-Tordo, D., and Lazdunski, M. (1975) J. Biol Chem. 250, 6040–6045) alkaline phosphatase, than to previously reported values for chicken (Cyboron, G. W., and Wuthier, R. E. (1981) J. Biol. Chem. 256, 7282–7268) and fetal calf (Fortuna, R., Anderson, H. C., Carty, R. P., and Sajdera, S. W. (1980) Calcif. Tissue Int. 30, 217–225) cartilage matrix vesicle alkaline phosphatase. The purified alkaline phosphatase was activated by micromolar Mg**. The amino acid composition of cartilage alkaline phosphatase was found to be similar to that previously described for porcine kidney (Wachsmuth, E. D., and Hiwada, K. (1974) Biochem. J. 141, 273–282). Double immunoprecipitation data indicated that monoclonal antibody against cartilage alkaline phosphatase cross-reacted with fetal bovine liver or kidney enzyme but failed to react with calf intestinal or rat cartilage enzyme. Thus these observations suggest that alkaline phosphatase of matrix vesicles from calcifying epiphyseal cartilage is a liver-kidney bone isozyme.

Alkaline phosphatase of calcifying tissues has been implicated in the initial calcification of cartilage, bone, and dentine (1). Matrix vesicles which are enriched in alkaline phosphatase, Ca binding factors, and enzymes that degrade various inhibitors of hydroxyapatite formation, are directly involved in the initiation of calcification in various tissues (for review see Ref. 2). To fully understand the role of matrix vesicles in calcification, each relevant component of the matrix vesicle must be identified, purified, and characterized. Although the purification and characterization of alkaline phosphatase of matrix vesicles from chick and bovine epiphyseal cartilage have been recently reported, several discrepancies with respect to its response to Mg** and its subunit molecular weight have emerged (3, 4). These discrepancies could be due to species difference, the use of different methods for vesicle isolation (4), or other unknown factors. Furthermore, earlier biochemical data indicate that a subunit molecular weight for matrix vesicle alkaline phosphatase is not typical for a liver-kidney-bone type enzyme (3, 4). To resolve these paradoxical observations, and to provide amino acid composition data which have yet to be reported for bone or cartilage alkaline phosphatase, we purified the enzyme from fetal bovine matrix vesicles by specific monoclonal antibody affinity chromatography and partially characterized the purified enzyme.

EXPERIMENTAL PROCEDURES

Matrix Vesicle Isolation

The extracellular matrix vesicle fraction was prepared from fetal bovine epiphyseal cartilage according to the method of Hsu and Anderson (5). This procedure was a modification of the original method of Ali et al. (6).

Epiphyseal growth plates were removed aseptically, minced into 3 to 5-mm pieces, and then digested in a solution (10 ml/g of tissue) containing 1,000 units/ml of crude collagenase (Sigma, Type I), 0.12 M NaCl, 0.01 M KCl, 1,000 units of penicillin/ml, 1 mg of streptomycin/ml, and 0.02 M Tris buffer, pH 7.45. The digestion was carried out at 37 °C for 3 h. The digest was centrifuged at 30,000 × g for 10 min, and the resulting precipitate was discarded. The supernatant was spun at 300,000 × g for 20 min and the resulting precipitate was washed once by resuspension with 10 mM Tris-buffered saline, pH 7.6. The final matrix vesicle precipitate was resuspended in a small volume of Tris-buffered saline.

* This research was supported by Research Grant DE 05262 from the National Institutes of Health. A preliminary report of these results has appeared as Abstract 3722 in the Abstracts of the 75th and 68th Annual Joint Meetings (1984) of the American Society of Biological Chemists and the American Association of Immunologists, respectively. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: Tes, N-tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N,N'-tetraacetic acid.
Purification of Matrix Vesicle Alkaline Phosphatase

A monoclonal antibody which reacted specifically with bovine matrix vesicle alkaline phosphatase was produced by a murine hybridoma, 82-13/4B3.4.2 (7). Ascites were harvested approximately 2-3 weeks following injection of 1 x 10^7 hybridoma cells into the peritoneal cavity of pristane primed BALB/c mice. The ascite fluid was clarified by centrifugation at 300,000 x g for 20 min. The antibody was then precipitated by addition of a saturated ammonium sulfate solution to a final 50% concentration. The precipitate was dissolved in 0.01 M Tris, 0.02% NaN₃, pH 7.5, and dialyzed exhaustively against the same solution. Approximately 3 ml of this material was applied to a 1.5 x 10-cm column of DEAE-Sephadex A50 (Pharmacia) pre-equilibrated in the same buffer. Protein was eluted stepwise by increasing NaCl concentrations at 5 mM increments. A peak fraction containing about 10 mg of protein was eluted with 0.01 M Tris, 0.1 M NaCl, and 0.02% NaN₃ (pH 7.5). This fraction was concentrated by ultrafiltration over an Amicon PM-30 membrane and was analyzed by immunoelectrophoresis using a panel of goat anti-mouse antisera which reacts with whole mouse serum and specifically detects mouse immunoglobulin heavy chains. The antisera were generously provided by Dr. Richard Assofsky, National Institutes of Health. The peak fraction containing IgG, monoclonal antibody judged to be approximately 90% pure with a major contaminant having the electrophoretic mobility of transferrin.

Preparation of Monoclonal Antibody Affinity Column

The purified monoclonal antibody was coupled to CNBr-Sepharose (Sigma) at a ratio of approximately 5 mg of protein/ml final bed volume, according to the manufacturer's instructions. In essence, the gel was hydrated in 1 mM HCl, pelleted by centrifugation, and mixed with the monoclonal antibody at 5 mg/ml in 0.1 M carbonate, 0.5 M NaCl, pH 8.6. The reaction was continued overnight at 4 °C with constant rotation. Unreacted sites were blocked with 0.1 M ethanolamine for 2 h at ambient temperature and subjected to 3 wash cycles consisting of alternating between 0.1% carbonate buffer, 0.5 M NaCl, pH 8.6, and 0.1 M acetic acid buffer, 0.5 M NaCl, pH 3.0. The column was then packed and equilibrated in 0.05 M Tris, 0.15 M NaCl, and 0.02% NaN₃, pH 8.0.

Enzyme Purification

Unless stated otherwise, all steps of purification were performed at 4 °C.

Step 1. Solubilization—One-fourth volume of n-butanol was added to matrix vesicle suspension (2 mg of protein/ml) in 10 mM Tris-buffered saline, pH 7.6. The mixture was vortexed vigorously and then centrifuged at 300,000 x g for 20 min. The supernatant was collected and the precipitate was discarded.

Step 2. Monoclonal Antibody Affinity Chromatography—Approximately 1 ml of the butanol extract (25.7 mg of protein) was then applied to a monoclonal antibody affinity column (0.7 x 7 cm), which had been equilibrated with 2% Tween 20, 10 mM Tris, pH 7.6. The column was then washed with 35 ml of the Tween-Tris buffer at 4 °C to remove unbound protein. The bound alkaline phosphatase was eluted with 15 ml of 0.6 M 2-amino-2-methyl-1-propanol, pH 10.2.

The active fractions were pooled, washed twice with 10 mM Tris, pH 7.6, and concentrated to 1-ml volume by filtration of buffer through an Amicon YM-30 filter.

Step 3. DEAE-cellulose Chromatography—The concentrated eluate from the affinity column was applied to a DEAE-cellulose column (0.7 x 7 cm) pre-equilibrated with 10 mM Tris-HCl, pH 7.6. The enzyme was then eluted from the column at 4 °C using a linear gradient between 10 mM Tris-HCl, pH 7.6, and a mixture of 10 mM Tris, pH 7.6, 1 M NaCl, 20 mM dodecylsulfate, and 10.7% butanol. The inclusion of dodecylsulfate and butanol in the second gradient significantly increased the yield of the enzyme. One-mI fractions were collected and assayed for absorbance at 280 nm and enzyme activity.

Alkaline Phosphatase Assay

The enzyme was assayed in 1-ml cuvettes by measuring the release of p-nitrophenol from p-nitrophenylphosphate at ambient temperature using a Gilford (Model 250) recording spectrophotometer. The assay mixture (0.5 ml) contained 0.3 M 2-amino-2-methyl-1-propanol, pH 10.2, 4 mM p-nitrophenylphosphate, 20 µM MgCl₂, and 10 µl of diluted enzyme. The formation of p-nitrophenol was determined spectrophotometrically at 410 nm.

Protein Determination

Protein concentrations were determined by the method of Lowry et al. (8) using bovine serum albumin as a standard.

SDS Slab Gel Electrophoresis

Polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method of Laemmli (9). Proteins were stained with silver according to the method of Merrell et al. (10).

Double Immunoprecipitation Assay

The cross-reactivity of anti-matrix vesicle alkaline phosphatase antibody with matrix vesicle alkaline phosphatase and kidney alkaline phosphatase was determined by double immunoprecipitation. Kidney was homogenized, rather than digested with collagenase, and extracted with butanol as described above. Aliquots of 25 µl of the butanol extract (about 1 mg of protein/ml) from matrix vesicles or kidney were added to 150 µl of 2% Tween, 10 mM Tris, pH 7.6. A one-fourth dilution of monoclonal anti-cartilage matrix vesicle alkaline phosphatase antibody was made and 10 µl was added to each reaction tube and incubated at 37 °C for 1 h. A small aliquot (10 µl) was removed for total activity before centrifugation at 9000 rpm for 5 min. Supernatants were collected and precipitates were rinsed twice with 10 mM Tris-buffered saline (pH 7.6) and resuspended in 250 µl of 10 mM Tris-buffered saline. Alkaline phosphatase activity was then measured from both supernatant and precipitate resuspension. The addition of monoclonal antibodies to the butanol extracts did not affect the enzyme activity. The enzyme bound to monoclonal antibody was then taken as the ratio of enzyme activity present in the precipitate (or remaining activity not present in the supernatants) to the total activity measured prior to centrifugation.

SDS Slab Gel Electrophoresis

Polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method of Laemmli (9). Proteins were stained with silver according to the method of Merrell et al. (10).
Duplicate aliquots of 30 μg of protein were digested in 6 N HCl at 100 °C for 24 h. The amino composition was determined in a Dornex D-300 Amino Acid Analyzer.

RESULTS

Purification

As shown in Fig. 1, a single application of the butanol extract of matrix vesicles to the monoclonal antibody affinity column caused a complete absorption of alkaline phosphatase whereas 99% of nonspecific proteins were not retained. Several eluting agents, including acid pH, high ionic strength, Mg^2+, and 2-amino-2-methyl-1-propanol, were tested for their effectiveness in eluting the enzyme from the insolubilized antibodies (Table I). Among these agents, 0.6 M 2-amino-2-methyl-1-propanol (pH 10.2) was the most effective agent. There is little absorbance at 280 nm in fractions corresponding to alkaline phosphatase activity. Presumably this indicates the high level of purity of the enzyme since amino acid analysis demonstrates the presence of aromatic acids (Table IV). Depending on the experiment, 80-100% of the enzyme effectiveness in eluting the enzyme from the insolubilized antibodies (Table I). Among these agents, 0.6 M 2-amino-2-methyl-1-propanol (pH 10.2) was the most effective agent. There is little absorbance at 280 nm in fractions corresponding to alkaline phosphatase activity. Presumably this indicates the high level of purity of the enzyme since amino acid analysis demonstrates the presence of aromatic acids (Table IV). Depending on the experiment, 80-100% of the enzyme was eluted by 2-amino-2-methyl-1-propanol. The enzyme was also very stable in the presence of this propanol derivative. The effectiveness of the organic compound may be attributed to the combination of high ionic strength, alkaline pH, and hydrophobic conditions. It should be noted that in contrast to liver alkaline phosphatase (11) the cartilage enzyme was not eluted by 3 M NaCl. In spite of extensive washing of the preparation using repeated Amicon filtration or dialysis, both Tween 20 and 2-amino-2-methyl-1-propanol were not effectively removed. The presence of these substances interfered with SDS-polyacrylamide gel electrophoresis and the protein stain. However, since they are nonanionic substances, we found that they could be removed effectively by sequential application to DEAE-cellulose (Fig. 2) and Sepharose CL-6B chromatography (Fig. 3). As shown in Fig. 2, a flow-through fraction appears to have a strong absorbancy at 280 nm (0.6 M 2-amino-2-methyl-1-propanol buffer and 2% Tween had A_{280} of 0.3 and 0.2, respectively). It should be noted that elution with 1 M NaCl greatly enhanced the recovery of the enzyme from the DEAE-cellulose column. Finally,

![Graph](image-url)

**TABLE I**

<table>
<thead>
<tr>
<th>Agents</th>
<th>Recovery</th>
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<tr>
<td>2-Amino-2-methyl-1-propanol, 0.6 M</td>
<td>80-100</td>
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<tr>
<td>MgCl_2, 1 M</td>
<td>25</td>
</tr>
<tr>
<td>NaCl, 3 M</td>
<td>1</td>
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<tr>
<td>Acetate buffer, pH 4.0, 0.2 M</td>
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<td>Urea, 6 M</td>
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**TABLE II**

<table>
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<tr>
<th>Step</th>
<th>Total activity</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Purification</th>
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<tr>
<td>Matrix vesicles</td>
<td>204</td>
<td>27.7</td>
<td>7.4</td>
<td>100</td>
<td>1</td>
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<tr>
<td>Butanol extract</td>
<td>200</td>
<td>16.8</td>
<td>11.9</td>
<td>98</td>
<td>1.6</td>
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<td>Affinity chromatography</td>
<td>201</td>
<td>0.326</td>
<td>616</td>
<td>99</td>
<td>82</td>
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<tr>
<td>DEAE-cellulose chromatography</td>
<td>157</td>
<td>0.245</td>
<td>640</td>
<td>77</td>
<td>86</td>
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<tr>
<td>Sepharose CL-6B chromatography</td>
<td>105</td>
<td>0.122</td>
<td>860</td>
<td>51</td>
<td>116</td>
</tr>
</tbody>
</table>

**Fig. 2.** DEAE-cellulose chromatography of pooled eluates from the affinity column. The concentrated eluate (1 ml) from the affinity column was applied to a DEAE-cellulose column (0.7 x 7 cm) pre-equilibrated with 10 mM Tris-HCl, pH 7.6. Alkaline phosphatase was then eluted from the column with a linear gradient between 50 ml of 10 mM Tris-HCl (pH 7.6) and a mixture of 50 ml of 1 M NaCl, 20 mM deoxycholate, 10.7% butanol, 10 mM Tris, pH 7.6. The active fractions (18 through 32) were pooled, concentrated, and washed twice with 5 ml of 10 mM Tris (pH 7.6) using an Amicon YM-30 filter, alkaline phosphatase; x, absorbance at 280 nm.

**Fig. 3.** Sepharose CL-6B chromatography. One ml of the concentrated eluate from the DEAE-cellulose column was applied to a Sepharose CL-6B column (1 x 21 cm) pre-equilibrated with 10 mM Tris, pH 7.6. The enzyme was subsequently eluted from the column with the same buffer. The active fractions (16 through 29) were pooled, concentrated, and washed twice with 5 ml 10 mM Tris (pH 7.6) using an Amicon YM-30 filter, alkaline phosphatase; x, absorbance at 280 nm.
Purification of Matrix Vesicle Alkaline Phosphatase

1829

A

1 2 3 4 5 6 7 8 9 10

B

1 2 3 4 5 6 7 8 9

FIG. 4. Slab gel electrophoresis of purified or partially purified alkaline phosphatase from fetal calf and rachitic rat matrix vesicles. The gels contained 7.5% acrylamide, 0.2% bisacrylamide, and 0.1% SDS. Protein samples were denatured with 1% SDS and 5% mercaptoethanol prior to electrophoresis (9). Gel electrophoresis was performed according to the method of Laemmli (9). Gels were stained with silver according to the method of Merril et al. (10). A, lanes 3, 4, and 10, Bio-Rad standard kit containing 5 µg each of the following proteins, myosin (M, = 200,000), galactosidase (116,000), phosphorylase b (92,500), albumin, (66,200), and ovalbumin (45,000); lanes 2 and 9, protein standards (Sigma) containing 5 µg each of myosin, galactosidase, albumin, ovalbumin; lane 5, 5.8 µg of fetal calf matrix vesicle alkaline phosphatase purified by sequential monoclonal antibody affinity, DEAE-cellulose, and Sepharose CL-6B chromatography; lane 6, 6.1 µg of rat matrix vesicle alkaline phosphatase partially purified by sequential Sepharose CL-6B, DEAE-cellulose, Sepharose CL-6B chromatography; lane 7, 54 µg of SDS solubilized calf matrix vesicles; lane 8, 50 µg of SDS-solubilized rachitic rat matrix vesicles; lane 1, sample buffer without protein. B, lanes 1, 5, and 9, Sigma standards; lane 2, 11.6 µg of fetal calf alkaline phosphatase, purified by sequential monoclonal antibody affinity, DEAE-cellulose, and Sepharose CL-6B chromatography; lanes 3 and 4, 54 µg of SDS-solubilized whole fetal calf matrix vesicles; lanes 7 and 8, 50 µg of SDS-solubilized rachitic rat matrix vesicles. C, calibration curve for molecular determination of matrix vesicle alkaline phosphatase. The five marker proteins are Bio-Rad standard kit containing: ●, myosin; ■, galactosidase; ▲, phosphorylase b; ○, albumin; △, ovalbumin. The extrapolated value for matrix vesicle alkaline phosphatase (×) is 81,000. The mobility values of various proteins were obtained from lanes 3 and 5 of A.

a Sepharose CL-6B column was used to remove any remaining traces of detergent in the eluate. This final chromatographic step caused a large loss in enzyme activity, which may have been due to nonspecific adsorption, removal of cofactors, or instability of the enzyme.

A typical purification is summarized in Table II. The alkaline phosphatase was purified 114-fold with a specific activity of 860 units/mg of protein.

Characterization

Molecular Weight Estimated by SDS-Polyacrylamide Gel Electrophoresis—On a 7.5% polyacrylamide-0.1% SDS gel,
Table III

<table>
<thead>
<tr>
<th>Preparations</th>
<th>Substrate</th>
<th>( \text{Mg}^{2+} ) or ( \text{Ca}^{2+} )</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affinity (fetal calf)</td>
<td>PNPPa</td>
<td>( \text{Mg}^{2+} ) 350 ± 45 (5)</td>
<td>% control</td>
</tr>
<tr>
<td>Affinity (fetal calf)</td>
<td>ATPb</td>
<td>( \text{Mg}^{2+} ) 568 ± 20 (3)</td>
<td></td>
</tr>
<tr>
<td>Affinity (fetal calf)</td>
<td>ATPb</td>
<td>( \text{Ca}^{2+} ) 108 ± 3 (3)</td>
<td></td>
</tr>
<tr>
<td>Conventional (fetal</td>
<td>PNPPa</td>
<td>( \text{Mg}^{2+} ) 265 ± 30 (3)</td>
<td></td>
</tr>
<tr>
<td>calf(^d)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conventional</td>
<td>PNPPa</td>
<td>( \text{Mg}^{2+} ) 270 (1)</td>
<td></td>
</tr>
</tbody>
</table>

The purified protein (in the presence of 1% SDS and 5% mercaptoethanol) migrated as a dominant band with an estimated molecular weight of 81,000 (Fig. 4, A and B). When 11 \( \text{mg} \) of purified enzyme was applied to SDS gels of both rat and fetal calf whole vesicle proteins, the major proteins of SDS-solubilized whole matrix vesicles from either fetal calf or rat were found to be in a molecular weight range of 20,000 and 33,000. However, a faint but recognizable band corresponding to the position of the pure enzyme was found in SDS gels of both rat and fetal calf whole vesicle proteins.

\( \text{Mg}^{2+} \) Effect—Table III and Fig. 5 show that the enzyme was activated by micromolar amounts of magnesium ion. For example \( \text{[Mg}^{2+}\] at 20 \( \mu \text{M} \) stimulated 2- to 3-fold. Indeed, an enzyme partially purified by gel filtration and subsequent anion exchange chromatography also was stimulated 2-fold by 20 \( \mu \text{M} \) \( \text{Mg}^{2+} \) (detail of purification not described). However, \( \text{Mg}^{2+} \) exerted no effect on the enzyme associated with intact matrix vesicles, probably indicating the presence of endogenous \( \text{Mg}^{2+} \) in the matrix vesicles (26).

Amino Acid Composition—The amino acid composition of the purified enzyme is shown in Table IV. The fetal calf cartilage enzyme is relatively enriched in glutamic and aspartic acids and the composition is very similar to that of swine kidney and calf intestinal enzymes (12, 13). The calf intestinal enzyme has more arginine and less histidine residues than fetal calf cartilage or swine kidney enzyme. The fetal calf cartilage enzyme has somewhat fewer alanine and valine residues than those of swine kidney or calf intestinal enzyme. These variations may be attributed to tissue as well as species differences.

**DISCUSSION**

Based on immunological characteristic, kinetics, and peptide analysis, three types of alkaline phosphatase have been classified, intestinal, placental, and liver-kidney-bone isoenzymes (14). Although bone and epiphyseal cartilage are both calcified tissues, the alkaline phosphatase of bone and cartilage, which may be an important part in the calcification mechanism, are not known to be biochemically identical. Several publications describing the purification of alkaline phosphatase of matrix vesicles have appeared since matrix vesicles were shown to be the initial site of calcification in many calcifying tissues (for review see Ref. 2). However, in these earlier studies the biochemical properties of the purified enzyme seemed to be significantly different from those already described for the liver-kidney-bone type of isozyme. For example, nonidentical subunits corresponding to molecular weights of 18,000 and 45,000, respectively, were described from fetal bovine epiphyseal cartilage (3) and a single subunit of 58,000 from chick cartilage was reported (4).

Since previously reported purification of alkaline phosphatase from matrix vesicles employed conventional methods of separation, we sought to improve the purification by using specific monoclonal antibody affinity chromatography. The new data utilizing a pure enzyme is at variance with earlier observations in suggesting that the molecular weight of fetal bovine cartilage alkaline phosphatase is similar to those of porcine kidney or rat liver alkaline phosphatase subunits which are in the vicinity of 80,000 (12-17). We previously reported data obtained from gel filtration and sucrose density gradient centrifugation which indicated that the cartilage matrix vesicle enzyme may exist as a dimer corresponding to
Purification of Matrix Vesicle Alkaline Phosphatase

a molecular weight of 160,000 (18). Similar results have been obtained with porcine kidney (12, 15).

The difference in the molecular weights of cartilage alkaline phosphatase purified by various laboratories may be due to species or tissue differences, the procedures for obtaining the enzyme-enriched matrix vesicles, or the chromatographic purification methods. It is not known to what extent the molecular weight of alkaline phosphatase is affected by proteolysis which may have occurred during crude collagenase digestion (19) or during the homogenization of cartilage (4). A specific selection by the monoclonal antibody affinity column of an alkaline phosphatase subset with a different molecular weight is unlikely, since about 90-100% of the enzyme was adsorbed by the column. In contrast to the observations of fetal calf (20) and chick epiphyseal cartilage (4), Stagni et al. (21) showed the presence of both soluble and intrinsic membrane forms of alkaline phosphatase in calf scapula cartilage. Further work is thus needed for the identification, purification, and characterization of various types of isozymes in the cartilage.

In addition to the Zn$^{2+}$ requirement for matrix vesicle alkaline phosphatase activity of fetal calf and chick cartilages (4, 18), purified chick alkaline phosphatase has been shown to be activated by Mg$^{2+}$ if the purified enzyme is pretreated with EGTA (4). Although Fortuna et al. (19) failed to observe Mg$^{2+}$ activation of alkaline phosphatase from fetal calf cartilage, the lack of activation may be due to the inclusion of Mg$^{2+}$ in an ion exchange column. Alkaline phosphatase from fetal bovine matrix vesicles purified by sequential applications of monoclonal affinity, DEAE-cellulose, and Sepharose CL-6B chromatography was activated 2-fold by micromolar ranges of Mg$^{2+}$. The Mg$^{2+}$ activation has also been observed in other mammalian tissues (17, 22-24). The difference in Mg$^{2+}$ activation in these instances may be attributed to the varying effectiveness of the purification procedure in removing Mg$^{2+}$ from the enzyme.

Similar to other purified phosphatases, the monoclonal affinity-purified enzyme was able to hydrolyze AMP, PP$\prime$, and ATP (data not shown). The pH optima for p-nitrophenylphosphate, ATP, AMP, and PP$\prime$ (data not shown) were also similar to the data obtained from the chick enzyme (4). It is interesting to note that alkaline phosphatase of chick epiphyseal cartilage may not function as a hydrolyzing enzyme under physiological conditions (25). Instead, it was proposed to be involved in P$\prime$ transport across the membrane. Whether the involvement of alkaline phosphatase in P$\prime$ transport across the membrane plays an important role in the initial calcification of cartilage remains to be established.

Double immunoprecipitation data (data not shown) indicated that the monoclonal antibody against cartilage matrix vesicle alkaline phosphatase cross-reacted with fetal bovine kidney enzyme but failed to react with calf intestinal or rat cartilage enzyme. Thus, we suggest that alkaline phosphatase of cartilage matrix vesicle is likely a liver-kidney-bone isozyme.

Although a rat liver alkaline phosphatase isoenzyme has been purified to homogeneity using monoclonal antibody affinity chromatography, the subunit molecular weight and amino acid composition were not reported and thus the liver alkaline phosphatase cannot be easily compared to cartilage enzyme (11). Liver alkaline phosphatase, in contrast to cartilage enzyme, was easily eluted from the affinity column with 3 M NaCl, but this difference may reflect variation in affinity constants with different antibody preparations. It does not necessarily indicate a true difference in the molecular structure of these enzymes.

Acknowledgments—We greatly appreciate the assistance of D. Vertz and B. Eklund in the preparation of this manuscript.

REFERENCES