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AFFINITY ELUTION FROM A PHOSPHONIC ACID–SEPHAROSE DERIVATIVE IN THE PURIFICATION OF HUMAN LIVER ALKALINE PHOSPHATASE

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SUMMARY

The compound *p*-aminobenzylphosphonic acid has been coupled via an azo linkage to tyraminyl–Sepharose 4B. This derivative at pH 6.0 bound most of the protein and all of the alkaline phosphatase in a crude preparation from human liver. The phosphatase was selectively eluted with the substrate 2-naphthylphosphate and a purification of 400-fold obtained. This step, when incorporated into a procedure for the purification of human liver alkaline phosphatase, yielded essentially pure enzyme.

INTRODUCTION

Comparative studies on the molecular properties of alkaline phosphatase* from tissues have been severely hampered by the lack of pure enzyme preparations and the small amounts of protein obtained. Recently, several procedures have been published[1–5] for the purification of human liver alkaline phosphatase but reported recoveries are low. In this paper we describe the synthesis of a phosphonic acid derivative of Sepharose which non-selectively binds protein. The conditions for biospecific elution of alkaline phosphatase were determined and the procedure was applied to the purification of human liver alkaline phosphatase. Landt *et al.*[6] have recently described the affinity chromatography, and inhibition by phosphonic acids, of bovine intestinal alkaline phosphatase.

EXPERIMENTAL

Materials

General chemicals were of reagent quality and were supplied by Fisher Scientific (Fair Lawn, N.J., U.S.A.). Biochemicals were generally of the highest purity available and were from the following sources: *p*-nitrophenylphosphate, 2-glycerophosphate, 2-naphthylphosphate, naphthol AS-MX phosphate, tyramine, D(+)-

* Enzyme = orthophosphoric monoester phosphohydrolase (alkaline optimum) (E.C. 3.1.3.1).

mannose, 2-amino-2-methylpropan-1-ol and Fast Blue BB salt (Sigma, St. Louis, Mo., U.S.A.); acrylamide, methylenebis(acrylamide), and Coomassie Blue (Bio-Rad Labs., Richmond, Calif., U.S.A.); Ampholine (LKB, Stockholm, Sweden); Mes [2-(N-morpholino)ethanesulphonic acid] (Calbiochem, Los Angeles, Calif., U.S.A.); neuraminidase (*Clostridium perfringens*, 6 units/mg) (Worthington Biochemical Corp., Freehold, N.J., U.S.A.); diethyl *p*-aminobenzylphosphonate, cyanogen bromide and phenylphosphonic acid (Aldrich, Milwaukee, Wisc., U.S.A.); DEAE-Sephadex A-50, Sephadex G-200, G-25, Sepharose 4B and Concanavalin A–Sepharose (Pharmacia, Uppsala, Sweden).

Enzyme and protein assays

Alkaline phosphatase was assayed as described previously[2] at pH 10.3 and 30°. One unit of enzyme activity corresponds to the hydrolysis of 1 μmole of *p*-nitrophenylphosphate per min.

Protein concentrations were determined by measurement of the absorbance at 280 nm, assuming an $E^{1\%}_{1\,cm}$ of 10, or by the method of Schacterle and Pollack[7].

Synthesis of p-aminobenzylphosphonic acid and its coupling via an azo linkage to tyraminyl–Sepharose 4B

p-Aminobenzylphosphonic acid was prepared by hydrolysis of diethyl *p*-aminobenzylphosphonate. The diethyl ester (2.5 g) was refluxed for 8 h in 50 ml concentrated HCl. The product was evaporated to *ca.* 10 ml, neutralized to pH 4 with 5 *M* NaOH and the fine precipitate collected by centrifugation. The product was washed with water and then ethanol, and dried for 30 min at 110°. Sepharose 4B (25 ml) was activated as described by Cuatrecasas[8] with 6.25 g of CNBr. The pH was maintained at 11 by the addition of 4 *M* NaOH and the temperature kept at 20° by the addition of ice. After 12 min the reaction was terminated by the addition of a large amount of ice, and the suspension rapidly filtered off and washed with 350 ml of ice-cold 0.1 *M* Na_2CO_3 buffer, pH 10, which contained 40% N,N-dimethylformamide. Tyramine hydrochloride (1.3 g), dissolved in 40 ml of the same buffer, was quickly added to the suspension and mixed gently for 12 h at 4°. The tyraminyl–Sepharose was then filtered off, washed with the same buffer and then washed with 0.1 *M* Na_2CO_3 buffer, pH 10.0.

The phosphonic acid derivative of tyraminyl–Sepharose was prepared by reaction of diazotized *p*-aminobenzylphosphonic acid with tyraminyl–Sepharose. *p*-Aminobenzylphosphonic acid (0.2 g) was dissolved in 10 ml of 1 *M* HCl and chilled on an ice-bath. Sodium nitrite solution (490 mg in 1 ml) was added to the stirred solution at 0° over 1 min. After 8 min the mixture was added with gentle stirring to the tyraminyl–Sepharose suspended in 25 ml of 0.1 *M* Na_2CO_3, pH 10, in an ice-bath. The pH was then adjusted to 9.3 with NaOH and the solution mixed gently at 4° for 4 h. The product was washed with 600 ml of 0.1 *M* Na_2CO_3, pH 10, followed by the same volume of 0.1 *M* acetate buffer, 0.5 *M* NaCl, pH 4.0, and then 0.1 *M* Tris–HCl buffer, 0.5 *M* NaCl, pH 8.0. The gel was equilibrated with 10 m*M* Mes buffer at pH 6.0 and stored at pH 6.0. The amount of the phosphonic acid bound to Sepharose was estimated by digestion of the gel followed by phosphate analysis[9]. Aliquots of gel were analyzed, in addition to samples containing *p*-aminobenzylphosphonic acid or standard phosphate solutions.

The gel was regenerated by washing first with 0.1 *M* Tris–HCl containing 0.5 *M* NaCl, pH 9.0, and then in the same buffer without NaCl.

Preparation of human liver alkaline phosphatase

The preliminary steps in the purification were as described[2] and are summarized in Table I. They included butanol extraction, acetone precipitation, ion-exchange chromatography and Concanavalin A–Sepharose chromatography. The eluate from the latter column that contained the alkaline phosphatase was adjusted to pH 6.0 with 100 m*M* Mes, pH 5.0. The solution was then applied to a 9 × 0.9 cm column of the phosphonic acid–Sepharose derivative, prepared as described above. The column was equilibrated with a solution (pH 6.0) containing 10 m*M* Mes, 100 m*M* NaCl, 0.1 m*M* $MgCl_2$ and 0.02 m*M* $ZnCl_2$. The column was washed with two column volumes of buffer and eluted with 10 m*M* Mes, 0.1 m*M* $MgCl_2$, pH 6.0, buffer containing 25 m*M* 2-naphthylphosphate. The fractions containing alkaline phosphatase activity were pooled and N,N-dimethylformamide was added to a final concentration of 10% (v/v) to prevent the precipitation of 2-naphthol formed by the hydrolysis of 2-naphthylphosphate.

TABLE I

PURIFICATION OF HUMAN LIVER ALKALINE PHOSPHATASE

Purification procedure	*Total protein (mg)*	*Total enzyme units**	*Specific activity (units/mg)*	*Recovery of enzyme (%)*	*Relative purity*
Butanol-treated homogenate	22,600	6800	0.3	100	1
30–50% Acetone fractionation	4900	4200	0.9	62	3
DEAE-Sephadex	1300	4100	3.2	60	11
Concanavalin A–Sepharose	104	2600	25.5	39	85
Phosphonic acid–Sepharose**		1700		26	
Sephadex G-200	1.13	1500	1300	22	4300

* One unit of enzyme will hydrolyze 1 μmole of *p*-nitrophenylphosphate per min.

** The presence of 2-naphthylphosphate prevents quantitative determination of protein at this step.

The pool was concentrated by ultrafiltration in an Amicon stirred cell with a PM-10 membrane (Amicon, Lexington, Mass., U.S.A.), and applied to a 90 × 2.6 cm gel filtration column (Sephadex G-200) equilibrated with a solution of 100 m*M* Tris–HCl, 100 m*M* NaCl, 0.1 m*M* $MgCl_2$, 0.02 m*M* $ZnCl_2$, pH 7.6. The fractions containing alkaline phosphatase activity were pooled and concentrated by ultrafiltration. The enzyme preparation was stored at 4° in the presence of 10% (v/v) glycerol.

Polyacrylamide-gel electrophoresis

Analytical polyacrylamide-gel electrophoresis was run in 7.0% (w/v) acrylamide gels in a Tris–borate buffer, pH 9.5, according to the method of Green *et al.*[10]. Enzymatic activity was located visually after incubation with 2-naphthylphosphate and Fast Blue BB salt[11], or the fluorescent bands were photographed under UV light after incubation with 6 m*M* naphthol AS-MX phosphoric acid, 6 m*M* $MgCl_2$ and

0.1 m*M* $ZnCl_2$ in 0.8 *M* 2-amino-2-methylpropan-1-ol buffer, pH 9.8. Protein was stained with Coomassie Brilliant Blue G-250 (ref. 12).

Sodium dodecyl sulphate polyacrylamide-gel electrophoresis

The gels were prepared and subjected to electrophoresis as described by Weber and Osborn[13]. Alkaline phosphatase samples were denatured by incubation in a mixture of 1% (w/v) sodium dodecyl sulphate and 1% (v/v) 2-mercaptoethanol for 5 min at 100°. After electrophoresis the gels were washed with several changes of 12.5% (w/v) trichloroacetic acid and stained for protein as described above.

Isoelectric focusing

Electrofocusing was performed in 4% (w/v) polyacrylamide gels which contained 2% (w/v) pH 3.5–10 Ampholine as previously described[2]. The alkaline phosphatase activity was detected as fluorescent bands as described above, and protein was stained according to the procedure of Allen *et al.*[14].

RESULTS

Synthesis of the phosphonic acid–Sepharose derivative

p-Aminobenzylphosphonic acid was obtained in 91% yield after acid hydrolysis of diethyl *p*-aminobenzylphosphonate. The product was relatively insoluble at neutral pH but was soluble at acid or alkaline pH. *p*-Aminobenzylphosphonic acid was found to be a mixed-type inhibitor of liver alkaline phosphatase with a strong competitive component and weak non-competitive component. The K_i at pH 9.0 was 0.55 m*M*.

A highly substituted gel which contained 10 μmole phosphorus per ml of packed gel was obtained after reaction of diazotized *p*-aminobenzylphosphonic acid with tyraminyl–Sepharose. The probable structure of the derivative is given in Fig. 1. The gel was amber at pH 6, turned yellow at more acid pH and reddish brown at alkaline pH. The gel performed satisfactorily after storage at pH 6.0 and 4° for one year.

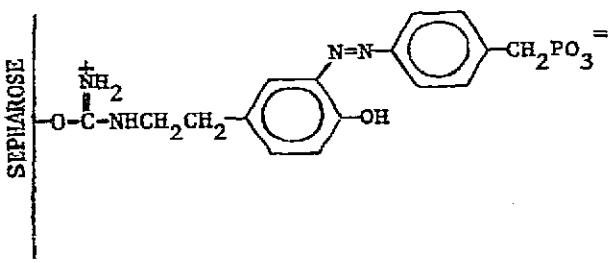


Fig. 1. Tyraminyl–Sepharose coupled via an azo linkage to *p*-aminobenzylphosphonic acid. This derivative is referred to in the text as phosphonic acid–Sepharose.

Elution of human liver alkaline phosphatase from the phosphonic acid–Sepharose derivative

Preliminary attempts failed to purify alkaline phosphatase by affinity chromatography with the phosphonic acid–Sepharose derivative because of non-specific

adsorption at pH 6.0. Conditions were therefore sought that would selectively elute the enzyme.

All of the adsorbed protein was eluted with 50 m*M* Tris–HCl, pH 9.0, in a single sharp peak. Ten column volumes of a linear gradient of 0–0.1 *M* phosphate, pH 6.0, failed to elute either alkaline phosphatase or other adsorbed protein. Less than 30% of the applied phosphatase was eluted with ten column volumes of a linear gradient of 0.1–1.0 *M* NaCl, pH 6.0. Hydrophobic interactions were not responsible for the adsorption of protein since 40 column volumes of a linear gradient of 0–50% (v/v) ethylene glycol, pH 6.0, failed to elute a significant amount of activity or protein.

Alkaline phosphatase was selectively eluted with 2-naphthylphosphate (Fig. 2). A 25-ml volume which contained very impure alkaline phosphatase at 0.7 units/mg protein in 10 m*M* Mes, 200 m*M* NaCl, pH 6.0, was applied to a 16 × 0.9 cm column of the phosphonic acid–Sepharose derivative equilibrated with the same buffer. Almost all of the applied protein and alkaline phosphatase were retained by the column, but the enzyme activity was eluted with 25 m*M* 2-naphthylphosphate in 10 m*M* Mes, pH 6.0; 89% of the applied activity was recovered in a volume of 240 ml. After concentration and dialysis to remove 2-naphthylphosphate, the specific activity was found to be 300 units/mg. This represents a 400-fold purification of the enzyme.

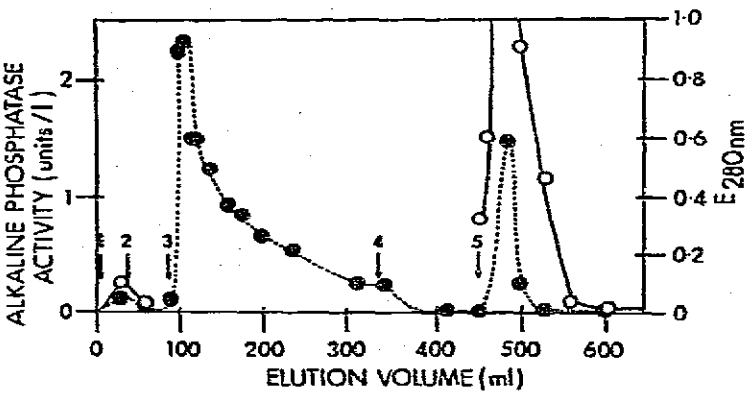
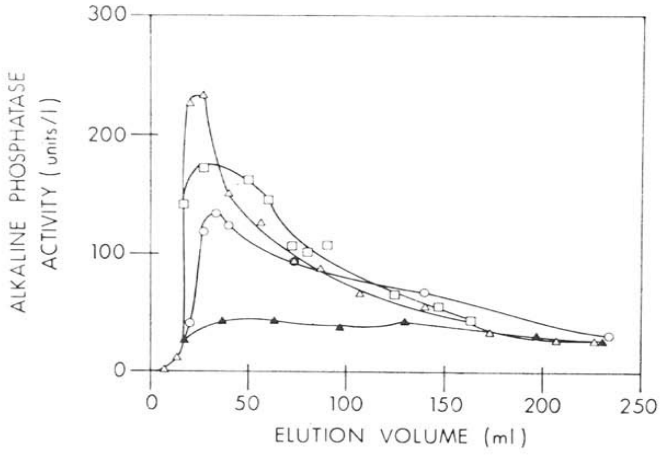


Fig. 2. Elution by 2-naphthylphosphate of human liver alkaline phosphatase from the phosphonic acid–Sepharose derivative. See text for details. 1, Start of sample application; 2, buffer wash; 3, elution with 10 m*M* Mes, 25 m*M* 2-naphthylphosphate, pH 6.0; 4, buffer wash; 5, regeneration with 50 m*M* Tris–HCl, pH 9.0. The fraction volume was 7.6 ml. The broken line represents the elution profile of alkaline phosphatase .The substrate, 2-naphthylphosphate, also absorbs strongly at 280 nm and interferes with the detection of protein (solid line). Essentially all of the protein impurities were present in the pH 9.0 eluate.

Other substrates and phosphonic acids also eluted the alkaline phosphatase activity and selected profiles are shown in Fig. 3. It is likely that the best eluant, 2-naphthylphosphate, has the highest affinity for the enzyme since the K_m values determined at pH 9.0 for 2-naphthylphosphate and 2-glycerophosphate were 5 μM and 56 μM respectively, and the K_i at pH 9.0 for phenylphosphonic acid was 75 μM (competitive inhibition). A combination of 10% (v/v) dimethylformamide and 180 m*M* NaCl eluted the enzyme relatively well (Fig. 3) but the eluate had a lower specific activity than that obtained by elution with 2-naphthylphosphate. Dimethylformamide



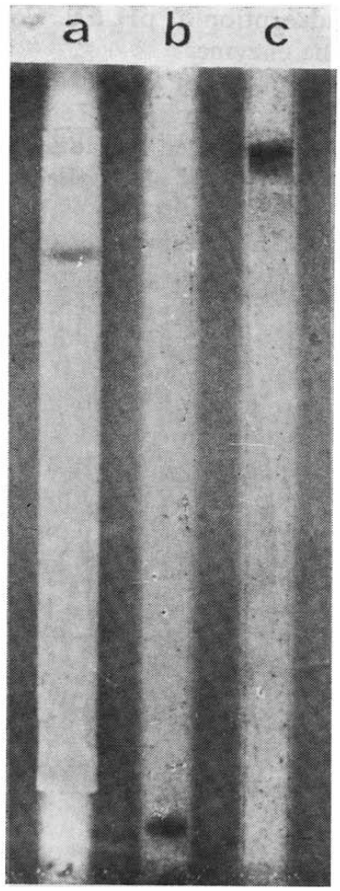


Fig. 3. Elution by various eluants of human liver alkaline phosphatase from the phosphonic acid–Sepharose derivative. The sample containing 35 mg protein and 23 units of alkaline phosphatase was loaded on a 16 × 0.9 cm column of the phosphonic acid–Sepharose equilibrated with 10 m*M* Mes, 200 m*M* NaCl, pH 6.0. The enzyme was eluted with 10 m*M* Mes at a final pH of 6.0 and contained either 25 m*M* 2-naphthylphosphate (△), or 10% (v/v) dimethylformamide and 180 m*M* NaCl (□), or 100m*M* 2-glycerophosphate (○) or 100 m*M* phenylphosphonic acid (▲).

Fig. 4. Sodium dodecyl sulphate (SDS) polyacrylamide-gel electrophoresis and isoelectric focusing of purified liver alkaline phosphatase. Proteins were stained with Coomassie Blue. a, SDS polyacrylamide-gel electrophoresis of 5 μg enzyme; b, isoelectric focusing with pH 3.5–10 Ampholine of 6 μg of enzyme; c, isoelectric focusing with pH 3.5–10 Ampholine of 6 μg of enzyme that had been treated with neuraminidase.

at 10% (v/v) considerably sharpened the profile of enzyme activity eluted with 25 m*M* 2-naphthylphosphate, but again the specificity obtained with the substrate alone was lost.

When the insoluble *p*-aminobenzylphosphonic acid was suspended in Sephadex G-25 (30 μmole/ml of packed gel) and an impure solution of alkaline phosphatase added, the enzyme was tightly bound and could be eluted under the conditions described above.

Purification of human liver alkaline phosphatase

A summary of a procedure in which the phosphonic acid biospecific elution step was incorporated into the purification of the enzyme is shown in Table I. Purity was assessed by sodium dodecyl sulphate polyacrylamide-gel electrophoresis, and isoelectric focusing of native (p*I* 3.9) and neuraminidase-treated enzyme (p*I* 6.7) on cylindrical polyacrylamide gels (Fig. 4)[2]. A minor protein band is present in the desialylated enzyme but both of these bands are enzymatically active. On the basis of these gels we judge the preparation to be greater than 95% pure.

DISCUSSION

The lack of sufficient quantities of pure enzyme has hampered the investigation of the tissue-specific isoenzymes of human alkaline phosphatases. The use of affinity elution from phosphonic acid–Sepharose, reported here, will provide a simple more efficient purification of the enzyme from liver. The procedure avoids the denaturation problems associated with preparative isoelectric focusing.

The reasons for the adsorption of proteins on the gel are uncertain. The gel contains both positive and negative charges and a high degree of hydrophobicity. Complete elution of alkaline phosphatase by alkaline buffers and partial elution by 1.0 *M* NaCl suggest that ionic interactions are involved, but the severe tailing may mean that hydrophobic attraction is also important. Ethylene glycol at 50% (v/v) did not elute the protein and hence hydrophobic bonding is not the major adsorption factor. Since alkaline phosphatase was retained by a column of Sephadex G-25 with insoluble *p*-aminobenzylphosphonic acid suspended in the gel, it is unlikely that the isourea and azo portions of the ligand (Fig. 1) play a significant role in the adsorption of the enzyme. Yon[15] has reported the non-selective binding of membrane proteins to a gel containing hydrophobic as well as positively and negatively charged groups, and he was able to elute with biospecific ligands. The best elution of alkaline phosphatase from our derivative was achieved using 2-naphthylphosphate, a substrate exhibiting a relatively high affinity for the enzyme. This suggests that the elution is biospecific.

Landt *et al.*[6] investigated several phosphonic acid columns for the adsorption and elution of alkaline phosphatase from bovine intestine. There was non-specific binding to D,L-tyrosyldiazobenzylphosphonic acid agarose but none to L-histidyldiazobenzylphosphonic acid agarose. Elution from all columns was accomplished with 10 m*M* phosphate, pH 8.0. Under their conditions, an 11-fold purification of the enzyme was achieved with the last mentioned derivative. Brenna *et al.*[16] and Komoda and Sakagishi[17] have reported on the use of immobilized phenylarsonic acid in the purification of alkaline phosphatase from intestinal tissue. However, this derivative had degraded after a few months of storage[18]. The immobilized benzylphosphonic acid is considerably more stable than this; we are still using a preparation that was synthesized 2 years ago. The inhibition constants for free *p*-aminobenzylphosphonic acid and free *p*-aminophenylarsonic acid are 0.55 m*M* and 3.8 m*M* respectively and this is reflected in a greater capacity for alkaline phosphatase of an immobilized derivative of *p*-aminobenzylphosphonic acid. We have synthesized several immobilized derivatives of *p*-aminophenylarsonic acid and all were inferior to the phosphonic acid derivative. Komoda and Sakagishi[17], using immobilized *p*-amino-

phenylarsonic acid, increased the specific activity of an alkaline phosphatase preparation from human intestine 31-fold in 30% yield. In the trial shown in Fig. 2, which involved alkaline phosphatase from human liver, a 400-fold purification was achieved and when the column was included as the penultimate step in the purification to homogeneity of the enzyme a 50-fold purification was obtained with a 67% yield (Table I).

Since the phosphonic acid–Sepharose gel binds most of the protein applied, it is necessary to remove the "bulk" of the protein before this step. Chromatography on DEAE-Sephadex and Concanavalin A–Sepharose were employed because of the relatively large purification factor achieved, with good recovery of alkaline phosphatase. The general procedure outlined in Table I has also been used to purify alkaline phosphatase from human intestine and kidney.

CONCLUSIONS

Alkaline phosphatase from human liver has been 95% purified by a procedure that involved affinity elution from tyraminyldiazobenzylphosphonic acid coupled to Sepharose 4B. The procedure is also suitable for other molecular forms of human alkaline phosphatase.

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