Affinity-Chromatography Purification of Alkaline Phosphatase from Calf Intestine

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A crude preparation of alkaline phosphatase (EC 3.1.3.1) from calf intestinal mucosa was purified by affinity chromatography on Sepharose-bound derivatives of arsanilic acid, which was found to be a competitive inhibitor of the enzyme. Three biospecific adsorbents were prepared for the chromatography, and the best results were obtained with a tyraminyl-Sepharose derivative coupled with the diazonium salt derived from 4-(*p*-aminophenylazo)phenylarsonic acid. Alkaline phosphatase was the only enzyme retained by the affinity column in the absence of P_i . The enzyme eluted by phosphate buffer had a specific activity of about 1200 units per mg of protein at pH 10.0, with 5.5 mM-*p*-nitrophenyl phosphate as the substrate.

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) catalyses the release of P_i from several phosphorylated compounds (Morton, 1965; Fernley, 1971).

The enzyme has been purified to various degrees after extraction from different animal tissues (Morton, 1954; Portmann, 1957; Engstrom, 1961, 1964; Behal & Center, 1965; Moss *et al.*, 1967; Ghosh & Fishman, 1968; Brunel *et al.*, 1969) and also from bacteria (Lazdunski & Lazdunski, 1967; Simpson *et al.*, 1968).

The enzyme from calf intestinal mucosa is one of the most active alkaline phosphatases. Its purification has been carried out so far by repeated fractionations with salts and organic solvents of the butan-1-ol extracts of the mucosa (Morton, 1954; Portmann, 1957), followed by conventional ion-exchange chromatography (Engstrom, 1961; Behal & Center, 1965).

We have investigated the purification of the enzyme from calf intestinal mucosa by affinity chromatography (Cuatrecasas *et al.*, 1968; Cuatrecasas, 1970, 1972), hoping to increase both the purity and the yield of the enzyme preparation.

We have used as selective adsorbents some derivatives of Sepharose 4B containing a phenylarsonic acid moiety. The choice of these compounds was based on the fact that inorganic arsenate has been reported to act as a competitive inhibitor of the enzyme from calf intestine (Morton, 1955).

Experimental

Materials

The following reagents were obtained from the suppliers shown: Sepharose 4B (Pharmacia, Uppsala, Sweden); CNBr and tyramine (Fluka A.G., Buchs,

Switzerland); arsanilic acid and chloroacetyl chloride (Carlo Erba, Milan, Italy); 4-(*p*-aminophenylazo)-phenylarsonic acid (Aldrich Europe, Beerse, Belgium) *p*-nitrophenyl phosphate (disodium salt); and 1,6-diaminohexane (BDH Chemicals, Poole, Dorset, U.K.). Reagent kit no. 85L for the specific stain of alkaline phosphatase was purchased from Sigma (London) Chemical Co., London S.W.6, U.K. Other reagents used were analytical grade.

Enzymic assays

Alkaline phosphatase, guanase, adenosine deaminase, nucleoside phosphorylase and inorganic pyrophosphatase were assayed as described in the *Biochemica Catalogue* (Boehringer, 1970). P_i determinations were by the method of Chen *et al.* (1956). Activity is expressed in units where 1 unit is the amount of enzyme that converts 1 μ mol of substrate/min at 25°C; specific activity is expressed as units/mg of protein. Alkaline phosphatase activity was assayed as a routine at pH 10.5 in 50 mM-sodium glycinate buffer containing 5.5 mM-*p*-nitrophenyl phosphate as substrate, 1 mM-MgCl₂ and 0.1 mM-ZnCl₂, but K_m and K_i were measured at the optimum pH (pH 10.0).

The assay conditions for alkaline phosphatase have often been different from those that are used here to define the unit. However, comparison with the results of other authors is possible on the following basis. Fernley (1971) quotes for Engstrom's preparation (Engstrom, 1961) the value of 2070 μ mol of substrate hydrolysed/min per mg of protein at 37°C under conditions of pH and substrate similar to those defined here. Engstrom reported that his preparation had the same specific activity as Portmann's (Portmann, 1957). The ratio of the alkaline phosphatase activities at 37°C and 25°C is about 1.6.

Protein concentration

This was determined by the method of Itzhaki & Gill (1964). At lower purification, concentrations were measured from an assumed specific extinction coefficient $E_{1\text{ cm}}^{1\text{ mg/ml}} = 1$ at 280nm. Absorbances were measured in a Beckman Acta III spectrophotometer.

Disc gel electrophoresis

The method of Davis (1964) was used except for the omission of the spacer gel. Solid sucrose (10%, w/v) was added to specimens before layering these on the top of the gels.

Coomassie Brilliant Blue R 250 (0.05% solution in acetic acid–propan-2-ol–water, 3:5:12, by vol.) was used for protein stain. Alkaline phosphatase activity was detected with Naphthol AS-MX and Fast Blue RR Salt (Sigma reagent kit no. 85L).

Enzyme extraction

Table 1 shows a scheme of the enzyme extraction. Duodenal calf intestine (2m) was cut out at the slaughter-house and placed on crushed ice. After the removal of fat, the intestines were chopped into 20cm pieces and these were cut open and washed with cold distilled water. The mucosa (25 ml/m of intestine) was gently scraped off the intestines with plastic knives, diluted with 1 vol. of 0.25M-sucrose and the mucosa was homogenized in a 4.5-litre Waring Blender for 2min at low speed.

The homogenate was extracted with 1.5ml of butan-1-ol per ml of original mucosa at 20°C with good mechanical stirring in a fermentor for 1h. The slurry was centrifuged at 4000g for 15min in a refrigerated Sorvall RC2-B centrifuge. The butanolic layer was removed by suction and the water extract filtered through cottonwool. The solid residue was re-extracted for 15min with 0.1M-KHCO₃. The slurry was centrifuged as above and the supernatant filtered through cottonwool. The pooled extracts were kept overnight at 4°C, centrifuged at 8000g for 20min and filtered through cottonwool.

The solution was then acidified slowly to pH 5 with 1M-acetic acid at 2°C with stirring. The precipitate

was spun down at 8000g for 20min and the pH of the cloudy supernatant was adjusted to 6.3 by the addition of solid KHCO₃.

Solid (NH₄)₂SO₄ was added to the cloudy solution to 40% saturation, and the suspension was spun at 4000g for 10min. The top layer of butan-1-ol formed by the salting-out action of (NH₄)₂SO₄ was removed by suction. More (NH₄)₂SO₄ was added to final 65% saturation with respect to the initial volume. The precipitate was removed by centrifugation as above and resuspended in 65% (w/v) (NH₄)₂SO₄ titrated to pH 6.5 with 1M-NH₃.

The overall yield of enzyme at this stage was 68% and the specific activity was about 20 units/mg of protein.

Preparation of specific adsorbents

Three different Sepharose derivatives (A, B and C) containing the phenylarsonic acid moiety were prepared (for chemical formulae see Fig. 1).

Derivative (A). Sepharose-bound tripeptide Gly-Gly-Tyr (Cuatrecasas, 1970) was treated with the diazonium salt of the arsanilic acid.

Derivative (B). Activated Sepharose [Axén *et al.* (1967) modified by Cuatrecasas (1970)] was treated with *N*-[*N*-(6-aminohexyl)glycyl]arsanilic acid, prepared as follows. *N*-Chloroacetylarsanilic acid (10mmol), prepared by the method of Giansa & Tropp (1926), was dissolved at 60°C in 100ml of ethylene glycol and added slowly over approx. 1h, with stirring, to 50ml of the same solvent containing 20mmol of 1,6-diaminohexane and 10mmol of triethylamine. The solution was heated at 60°C for 36h. Excess of 1,6-diaminohexane was removed by steam-distillation after the addition of 60ml of 0.5M-NaOH. The remaining solution was titrated to pH 5 with 2M-HNO₃ and the white precipitate was filtered, washed with cold distilled water, ethanol, diethyl ether and dried over CaCl₂ under vacuum. The product, *N*-[*N*-(6-aminohexyl)glycyl]phenylarsonic acid, was chromatographed in butan-1-ol–acetic acid–water (4:1:1, by vol.). Spots were detected as described by Haworth & Heathcote (1969) and by Rydon & Smith (1952) for the stain of amino and amido groups respectively.

Table 1. *Partial purification of calf intestine alkaline phosphatase*

Step	Volume (ml)	Activity (units)	Specific activity (units/mg of protein)	Yield (%)
Sucrose homogenate	700	91 000	—	100
Aqueous extracts before acidification	730	71 000	1.8	78
Neutralized extract	750	65 000	6	71
(NH ₄) ₂ SO ₄ ppt. 40–65% (w/v)	—	62 000	22	68

Fig. 1. Sepharose derivatives used for affinity chromatography of calf intestine alkaline phosphatase

The product was used without further purification since the observed 5% impurity contained amido groups but failed to give a positive ninhydrin test, suggesting that the impurity was the disubstituted derivative of 1,6-diaminohexane which could not interfere in the subsequent step. About 1g of this product was dissolved in 50ml of 0.1M- Na_2CO_3 buffer, pH 10, and the solution added to 50ml of Sepharose 4B activated with 12.5g of CNBr (Cuatrecasas, 1970), and the reaction mixture was gently stirred overnight at 4°C. The derivative obtained was filtered and washed to remove unbound *N*-[*N*-(6-aminoethyl)glycyl]phenylarsonic acid.

Derivative (C). This was prepared by coupling the diazonium salt of 4-(*p*-aminophenylazo)phenylarsonic acid to a tyramine-Sepharose derivative obtained by reaction of tyramine with activated Sepharose. The preparation was as follows. Packed Sepharose 4B (200ml) was activated as described by Cuatrecasas (1970) with 250mg of CNBr/ml of gel. The suspension was filtered and rapidly washed with 2 litres of ice-cold 0.1M- Na_2CO_3 buffer, pH 10.

A solution of 8.22g of tyramine in the carbonate buffer containing 40% (v/v) dimethylformamide, pH 10, was added to the activated Sepharose and the reaction mixture was gently stirred at 4°C overnight. The suspension was then filtered, washed with the carbonate buffer, followed by 40% (v/v) dimethylformamide and water. 4-(*p*-Aminophenylazo)phenylarsonic acid (1.6g) was dissolved in 80ml of 1M-HCl and the solution chilled in an ice bath; 0.5M- NaNO_2 solution (10ml) was added over a 10min interval. After an additional 15min period, the deep-brown solution was added to the tyramine-Sepharose suspended in 200ml of the carbonate buffer, the pH was adjusted to 9.3 and the reaction mixture moderately stirred for 5h at 4°C. The deep-brown Sepharose

derivative was washed with 5 litres each carbonate of buffer, 6M-urea, 25mM-HCl, 25mM-NaOH. The gel was then transferred into a chromatographic column and washed with distilled water until a clear effluent was obtained.

Analysis of arsenic

The content of bound inhibitor was estimated by determining the amount of arsenic present. For this purpose about 30ml of each derivative (A), (B) and (C) was thoroughly washed with 6M-urea, distilled water and 30, 60 and 95% (v/v) ethanol-water mixtures and finally dried under vacuum over CaCl_2 . About 1g of each dry derivative was obtained. Samples (0.1g) were treated as described by Kopp (1973) and arsenic analyses were carried out in an atomic-absorption spectrophotometer IL 151 equipped with an argon- H_2 flame, and with background correction. AsH_3 was generated by using NaBH_4 tablets (R. Bravi & M. Fittipaldi, unpublished work). H_3AsO_3 solutions were used as standards.

Results and Discussion

Choice of adsorbent

Table 2 summarizes the analytical data on the arsenic content of the three derivatives together with the corresponding gel capacity for retaining the alkaline phosphatase activity by biospecific adsorption. Inspection of the data shows that all three derivatives were successful in the biospecific binding of the intestinal enzyme, though they were different in the relative efficiency. They differed also in the specific affinity for alkaline phosphatase. In fact,

Table 2. Gel efficiency in affinity chromatography of calf intestine alkaline phosphatase

The gel efficiency is measured here as the ratio between the alkaline phosphatase units retained by 1 ml of gel and its arsenic content. The gel efficiencies are shown relative to the value obtained with derivative (B).

Derivative	Arsenic content		Alkaline phosphatase (units retained per ml of gel)	Relative efficiency
	($\mu\text{mol/g}$ dry wt.)	($\mu\text{mol/ml}$ of packed gel)		
A	12	0.6	10	38
B	43	2.2	1	1
C	153	7.6	40	12

Fig. 2. Chromatographic pattern of a crude preparation of calf intestinal alkaline phosphatase on a column (3 cm $\times$ 21 cm) packed with a tyraminyl-Sephadex derivative coupled with the diazonium salt of 4-(*p*-aminophenylazo)-phenylarsonic acid (derivative C)

Elution was started with 10 mM-Tris-HCl buffer, pH 8.4. Fractions of volume 12 ml were collected. At arrow (a) a 0.1 M Tris-HCl buffer, pH 8.4, was applied; at arrow (b) a linear gradient made with sodium phosphate (400 ml of the 0.1 M-Tris buffer, pH 8.4, +400 ml of the same buffer made 40 mM in phosphate at the same pH) was added. The content of tubes 67-70 was pooled. $\bullet$, E_{280} ; $\circ$, alkaline phosphatase activity.

adsorbent (A), although the most efficient, retained other proteins besides alkaline phosphatase. This probably occurred because the presence of a carbonyl group on the tyrosine residue gave the gel some ion-exchanger character. Adsorbent (B) was prepared to avoid these side effects. Alkaline phosphatase was the only enzyme retained by the column and eluted by P_i , but the amount of enzyme retained by this gel was poor in comparison with that of adsorbent (A) under similar conditions. This was probably due to the shortened spacer (1.5 nm instead of 2 nm as in the first derivative) or also to folding of the spacer in the absence of the rigid structure of the azobenzene group.

Adsorbent (C) was less efficient than adsorbent (A) but showed specific affinity for alkaline phosphatase.

Affinity chromatography

Fig. 2 shows the elution profile of enzyme from a column (3 cm $\times$ 21 cm) packed with adsorbent (C) and loaded with 2500 units of enzyme. The column and the enzyme samples were equilibrated with 10 mM-Tris, adjusted to pH 8.4 with 1 M-HCl, containing 1 mM-MgCl₂ and 0.1 mM-ZnCl₂.

Elution with the same buffer removed proteins with no alkaline phosphatase activity as shown by the first peak in Fig. 2. A tenfold increase in the Tris-HCl buffer molarity resulted in the elution of a little more inactive material (second peak in Fig. 2).

A linear gradient from 0.1 M-Tris-HCl, pH 8.4, to the same buffer containing 40 mM-sodium phosphate (molarity referred to phosphate) was applied and alkaline phosphatase was readily eluted.

The fractions containing alkaline phosphatase activity were pooled and assayed for activity and protein; a specific activity of 900 units/mg of protein was found (at pH 10.5).

As the enzyme solution was very dilute, some inactivation occurred during the enzyme concentration by ultrafiltration with the Amicon apparatus through a PM-30 Diaflo membrane. Thus the specific activity dropped to about 600 units/mg of protein.

Enzymic contaminants

In the crude preparation some contaminating activities were present to various extents. These were chiefly adenosine deaminase (2% of the phosphatase activity) and nucleoside phosphorylase (1.5%).

The purified enzyme had no adenosine deaminase and nucleoside phosphorylase, but retained about 7% inorganic pyrophosphatase activity. This was present also in the crude preparation. The pyrophosphatase activity was determined in the presence of 1 mM-MgCl₂.

This finding agrees with observations by Fernley & Walker (1967), Moss *et al.* (1967) and Brunel *et al.* (1969) on the pyrophosphatase activity of alkaline phosphatase from human intestine and liver and from bovine brain.

Fig. 3. pH-activity profile

Glycine-NaOH buffers ($I = 0.05 \text{ mol/l}$) at various pH values were prepared as described by Long *et al.* (1968). *p*-Nitrophenyl phosphate (5.5 mM) was present as the substrate and 0.1 mM-ZnCl₂ and 1 mM-MgCl₂ as co-factors.

Electrophoretic characterization of alkaline phosphatase

Disc gel electrophoresis in Tris-glycine buffer, pH 8.3, showed only two bands after staining the gels with Coomassie Blue, corresponding to two bands of enzymic activity. The slower-moving band had a specific activity 8–10 times that of the faster one. This approximate estimate was made by comparing the scans of the gel stained with Coomassie Blue with that obtained by specifically staining the gels for alkaline phosphatase activity. Similar observations have been reported for human intestinal alkaline phosphatase by Moss (1963).

Kinetic characterization of alkaline phosphatase

The pH-activity profile of the enzyme preparation is shown in Fig. 3. The activity maximum was found in the range 10–10.1 with, as the substrate, 5.5 mM-*p*-nitrophenyl phosphate in 50 mM-glycine-NaOH buffer containing 1 mM-MgCl₂ and 0.1 mM-ZnCl₂. Thus the specific activity usually measured at pH 10.5 was about 75% of the maximum.

The Michaelis constant at pH 10 was found to be $2.7 \times 10^{-4} \text{ M}$ (Fig. 4).

Inhibition by arsanilic acid at the optimum pH was found to be linearly competitive (Fig. 4, insert) and a K_i of $3.8 \times 10^{-3} \text{ M}$ was calculated.

Specificity of the adsorbent

Adsorbent (C) showed specific affinity for alkaline phosphatase since both electrophoresis and enzymic assays excluded the presence of detectable amounts

Fig. 4. Inhibition of calf intestine alkaline phosphatase by arsanilic acid

The assay was carried out at 25°C in 50 mM-sodium glycinate buffer, pH 10.0. The inhibitor was added as a 0.1 M solution of arsanilic acid which had been adjusted to pH 10 with 1 M-NaOH. ●, No inhibitor; ○, [I] = 2 mM; ■, [I] = 4 mM; □, [I] = 8 mM; ▲, [I] = 12 mM. In the absence of inhibitor $K_m = 2.7 \times 10^{-4} \text{ M}$. Insert: slope (K_m/V_{max}) plotted against inhibitor concentration (mM). A value of $3.8 \times 10^{-3} \text{ M}$ was obtained for K_i . The reaction rate is expressed as μmol of *p*-nitrophenol released/min per ml of enzyme solution at 25°C.

of contaminants besides the two proteins exhibiting alkaline phosphatase activity. The observed pyrophosphatase activity associated with alkaline phosphatase activity appears to support the above-mentioned observation of other investigators on the intrinsic pyrophosphatase properties of alkaline phosphatase.

As to the nature of the specific affinity of the adsorbent for alkaline phosphatase, this can probably be explained in terms of competitive binding of the arsonic acid moiety of the spacer to the enzyme. In fact, the bound enzyme was released by P_i at about 10 mM concentration. The arsenic content of the gel packed in the affinity column was also about 10 mM. Elution with 1 M-KCl resulted in a much slower release of the bound enzyme than that brought about by P_i. The above findings indicate that hydrophobic interactions and the ion-exchange effect of the arsonic group played a minor role in the affinity chromatography. This was confirmed also by using a gel which had a spacer similar to that of adsorbent (C) and a sulphonic acid group replacing the arsonic acid group. The amount of alkaline phosphatase activity retained by this column was 10% of that normally bound to the affinity column. Release of the enzyme was also brought about by a lower P_i concentration (3–4 mM).

Ion-exchange chromatography of the crude alkaline phosphatase preparation was carried out by

using a DEAE-cellulose column equilibrated with 10mM-Tris-acetic acid buffer, pH8.4, containing 0.5mM-magnesium acetate. Alkaline phosphatase was eluted with a linear gradient of Mg^{2+} (0.5–100mM) in the same Tris buffer. The specific activity of the eluted enzyme was about 130 units/mg of protein. Disc gel electrophoresis of the product revealed the presence of about 80% of protein contaminants. Adenosine deaminase was eluted very close to the alkaline phosphatase peak. Thus, to keep the amount of this contaminant less than 0.05% relative to the alkaline phosphatase, only 30% of the latter enzyme was recovered. The alkaline phosphatase showed about 10% of pyrophosphatase activity. These findings clearly prove the advantage of the affinity chromatography over the conventional ion-exchange chromatography because of the high specificity of the adsorbent used.

Conclusions

The value of specific activity of the enzyme obtained after the affinity-chromatography step is among the highest so far reported for the calf intestinal enzyme and is comparable with those reported by Portmann (1957) and Engstrom (1961), i.e. about 1200 units/mg of protein. The yield of the affinity-chromatography step is almost quantitative, since 80–90% of the activity is recovered. In this regard the method described here represents a considerable improvement over reported purifications of alkaline phosphatase from calf intestinal mucosa.

Doellgast & Fishman (1974) described the chromatography of human placental and calf intestinal alkaline phosphatase adsorbed on a column packed with an L-phenylalanine-Sepharose derivative. This chromatographic procedure is reported to increase the specific activity of the placental enzyme by a factor of 3. No details are given for the calf intestinal enzyme. The affinity-chromatography procedure described here causes a 50-fold increase in the specific activity of the intestinal enzyme. Doellgast & Fishman (1974) assume that the enzyme adsorption on the gel they prepared is of the hydrophobic type, whereas the procedure reported here is probably a true biospecific affinity-chromatography purification. In fact, the calf intestinal enzyme is almost exclusively the only species retained by Sepharose derivative (C), as judged by electrophoresis and assays of some enzymic contaminants, and elution can be easily attained by using a competitive inhibitor such as P_1 .

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