Biochem. J. (1974) **141**, 103–112
Printed in Great Britain

Purification of Human Placental Alkaline Phosphatase

SALT EFFECTS IN AFFINITY CHROMATOGRAPHY

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(*Received* 15 *October* 1973)

Human placental alkaline phosphatase was chromatographed on Sepharose derivatives of D- and L-phenylalanine, L-leucine, glycine, aniline and *p*-aminobenzoic acid in high concentrations of $(NH_4)_2SO_4$. Retention on these columns was greatest at the highest concentrations of $(NH_4)_2SO_4$. By using decreasing concentrations and changing the types of salts, elution was effected from each of the columns. The $(NH_4)_2SO_4$-mediated retention appeared to be related to the hydrophobic character of the substituted Sepharose, rather than to any specific binding site of the enzyme. It is suggested that this provides a way of controlling hydrophobic affinity chromatography. By use of chromatography on L-phenylalanine–Sepharose and of DEAE-Sephadex chromatography in the presence of Triton X-100 detergent, a preparation of highly purified (1000-fold) human placental alkaline phosphatase was obtained in 22% yield.

Doellgast & Kohlhaw (1972) reported that α-isopropylmalate synthase [EC 4.1.3.12; α-isopropylmalate α-ketoisovalerate-lyase (CoA-acetylating)] from *Salmonella typhimurium* could be retained on Sepharose derivatives of its feedback inhibitor, L-leucine, at high concentrations of potassium phosphate buffer, pH6.8. A similar result has been reported for threonine deaminase [EC 4.2.1.16; L-threonine hydro-lyase (deaminating)] and for acetohydroxyacid synthetase on columns of L-isoleucine–Sepharose (Brown *et al.*, 1972) and for biosynthetic threonine deaminase on isoleucine–, valine–, threonine– and leucine–Sepharose (Rahimi-Laridjani *et al.*, 1973).

As human placental alkaline phosphatase is uncompetitively inhibited by L-phenylalanine with a K_i of 4–5mM (Ghosh & Fishman, 1968), it is possible that the enzyme would bind specifically to L-phenylalanine–Sepharose as in the case of α-isopropyl-malate synthase to L-leucine–Sepharose. We therefore decided to ascertain whether this enzyme could be purified by using a similar effect of salts to promote its binding to L-phenylalanine–Sepharose. Since high concentrations of phosphate inhibit alkaline phosphatase, their use could interfere with subsequent assay of the enzyme, so it was convenient to use sulphate salts to chromatograph placental alkaline phosphatase on L-phenylalanine–Sepharose columns. Sulphate salts were found to have an effect similar to phosphate salts on retention of α-isopropylmalate synthase from *Salmonella typhimurium* on L-leucine–Sepharose (G. J. Doellgast & G. B. Kohlhaw, unpublished work).

By a sequence of L-phenylalanine–Sepharose chromatography at decreasing concentrations of $(NH_4)_2SO_4$, and DEAE-Sephadex chromatography in the presence or absence of Triton X-100 detergent, we were able to prepare a highly purified (1000-fold) placental alkaline phosphatase. The method of purification reported here results in a considerably higher yield of placental alkaline phosphatase than that reported previously (Ghosh & Fishman, 1968).

That the sulphate-mediated binding of placental alkaline phosphatase may be due to a non-specific interaction with phenylalanine–Sepharose was concluded from an investigation of the binding of placental alkaline phosphatases to Sepharose derivatives of glycine, leucine, D-phenylalanine, aniline and *p*-aminobenzoic acid at high concentrations of $(NH_4)_2SO_4$ and of alkaline phosphatases from other species on L-phenylalanine–Sepharose. Chromatography of proteins on Sepharose derivatives of certain amino acids and other organic residues may therefore be a generally useful purification technique for proteins.

Experimental Procedures

Materials

Chemicals used in this study and their suppliers were: phenyl phosphate, disodium salt (Sigma Chemical Co., St. Louis, Mo., U.S.A.); Tris and $(NH_4)_2SO_4$ ('ultra-pure' reagent, Mann, New York, N.Y., U.S.A.); Sepharose 4B and DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden); CNBr (Eastman

Organic Chemicals Dept., Rochester, N.Y., U.S.A.); Triton X-100 (Calbiochem, Los Angeles, Calif., U.S.A.); Ampholines (LKB, Stockholm, Sweden); aniline hydrochloride and *p*-aminobenzoic acid, sodium salt (Matheson, Coleman and Bell, East Rutherford, N.J., U.S.A.); *Escherichia coli*, calf intestinal, pig intestinal and chicken intestinal alkaline phosphatases (Sigma). All other chemicals were of the highest grade commercially available.

Enzyme assays

For alkaline phosphatase these were performed at 37°C with 18mM-phenyl phosphate in 50mM-sodium carbonate–sodium bicarbonate buffer (pH9.8)–10mM-$MgCl_2$ (Fishman *et al.*, 1972). The unit of enzyme activity is defined as that amount which liberates 1μmol of phenol/min per ml under these conditions.

Protein

This was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Sepharose derivatives

These were prepared by the procedure of Cuatrecasas (1970) by using 20g of CNBr/100ml (settled volume) of Sepharose in the activation step, and 0.2M-ligand at pH9.8 in the coupling step. For preparation of aniline–Sepharose, the coupling reaction was performed in 80% (v/v) acetone, in which Sepharose has

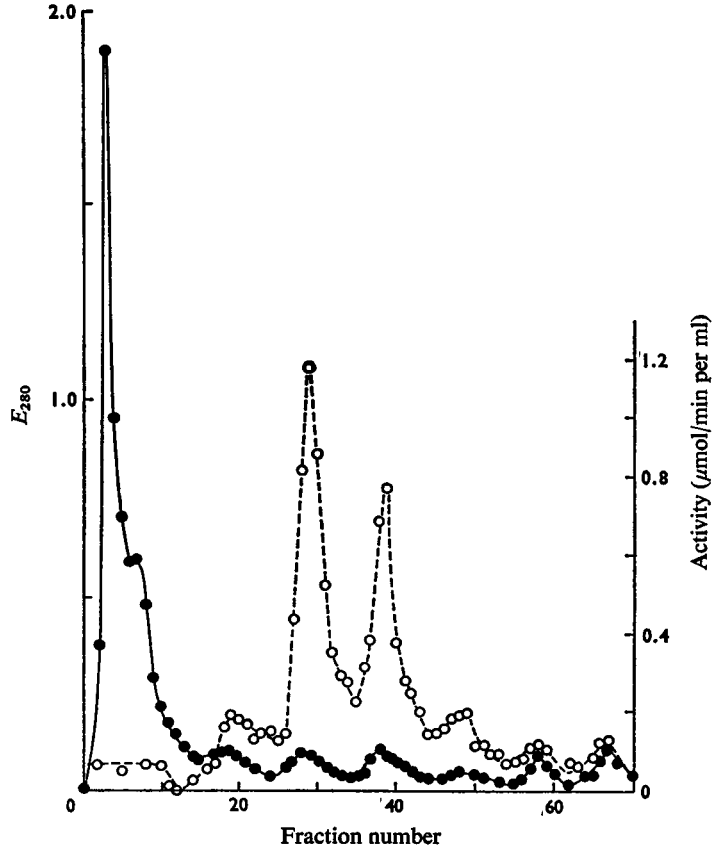


Fig. 1. *Elution profile of human placental alkaline phosphatase on* L-*phenylalanine–Sepharose*

A portion (0.5ml) of a butanol-treated dialysed sonicated extract of placenta containing 16.3mg of protein/ml and 20 units of enzyme activity, in 1.25M-$(NH_4)_2SO_4$–0.05M-Tris–acetate, pH8.0, was applied to a column (0.9cm×11.0cm) of L-phenylalanine–Sepharose. The column was washed successively with various media, as follows: fractions 1–15, 1.25M-$(NH_4)_2SO_4$–0.05M-Tris–acetate, pH8.0; fractions 16–25, 1.0M-$(NH_4)_2SO_4$–0.04M-Tris–acetate, pH8.0; fractions 26–35, 0.75M-$(NH_4)_2SO_4$–0.03M-Tris–acetate, pH8.0; fractions 36–45, 0.05M-$(NH_4)_2SO_4$–0.02M-Tris–acetate, pH8.0; fractions 46–55, 0.25M-$(NH_4)_2SO_4$–0.01M-Tris–acetate, pH8.0; fractions 56–64, 0.025M-NaCl–0.05M-Tris–acetate, pH8.0; fractions 65–70, 0.25M-Tris base, pH10.5. The elution was performed at 4°C; the fraction size was 2ml. ●, E_{280}; ○, activity.

been shown to be stable (Axen & Ernback, 1971). The gel was then washed exhaustively with acetone before being used for chromatography.

Disc gel electrophoresis

The method of Davis (1964) was used with 0.6cm× 8.5cm gels, without a spacer or sample gel, and with 20% (v/v) glycerol in the sample solution. Both sample and gel contained 0.5% Triton X-100 (Fishman, 1974).

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

The method of Fairbanks *et al.* (1971) was used.

Immunodiffusion experiments

These were carried out in 0.05M-veronal–HCl buffer, pH8.5, in 1.5% (w/v) agarose gels. Diffusion was allowed to proceed for 5 days at room temperature, and the gels were then washed with five changes of 0.9% NaCl over a period of several days before being stained for protein or enzyme activity.

Isoelectric focusing

Gels were prepared and run by the procedure of Righetti & Drysdale (1971).

All gels were stained for protein by the method of Fairbanks *et al.* (1971). Activity was determined on disc gels and isoelectric-focusing gels by using α-naphthyl phosphate and Variamine Blue, as previously reported (Fishman, 1974) and on immunodiffusion gels with 2mM-naphthol AS-MX phosphate in 50mM-carbonate–bicarbonate buffer, pH9.8, containing 10mM-$MgCl_2$, and photographing the released fluorescent naphthol AS-MX under u.v. light.

Results

Effects of salts on hydrophobic affinity

The effect of using decreasing concentrations of $(NH_4)_2SO_4$ on the elution profile of human placental alkaline phosphatase from L-phenylalanine–Sepharose is shown in Fig. 1. The enzyme is applied in 1.25M-$(NH_4)_2SO_4$ to a column equilibrated with 1.25M-$(NH_4)_2SO_4$. Successive elutions with 1.25M-, 1.0M-, 0.75M-, 0.50M- and 0.25M-$(NH_4)_2SO_4$, 0.25M-NaCl and 0.25M-Tris base resulted in most of the enzyme activity being eluted as two peaks in the 0.75M- and 0.50M-$(NH_4)_2SO_4$ elutions. The peak in tubes 2–4 represents material excluded from the column, and therefore having a high molecular weight, possibly nucleic acids.

To acertain whether the retention was related to the inhibition of the enzyme by L-phenylalanine, three sets of controls were set up.

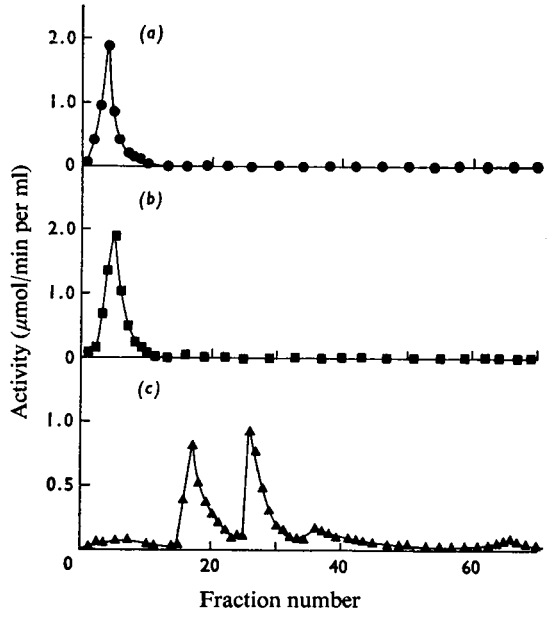


Fig. 2. *Elution profiles of human placental alkaline phosphatase on (a) unmodified Sepharose, (b) glycine–Sepharose, (c) L-leucine–Sepharose*

The same enzyme sample as was used in Fig. 1 was applied to columns with the following dimensions: unmodified Sepharose, 0.9cm×9.6cm; glycine–Sepharose, 0.9cm× 10.3cm; L-leucine–Sepharose, 0.9cm×10.1cm. Elution conditions were identical with those in Fig. 1.

First, unmodified Sepharose, glycine–Sepharose and L-leucine–Sepharose were compared for their retention of placental alkaline phosphatase. As seen in Fig. 2, the unmodified Sepharose and glycine–Sepharose both showed no significant retention of the enzyme in 1.25M-$(NH_4)_2SO_4$, but L-leucine–Sepharose retained the enzyme which was eluted in two main peaks, at 1.0M- and 0.75M-$(NH_4)_2SO_4$.

Secondly, D-phenylalanine–, aniline– and *p*-aminobenzoic acid–Sepharose were prepared and tested for their respective ability to retain the enzyme. The elution profiles of the enzyme on these three columns are shown in Fig. 3. The elution profile of the enzyme on D-phenylalanine–Sepharose was identical with that on L-phenylalanine–Sepharose (Fig. 1), which showed that the retention was not stereospecific. Further, the enzyme was retained very strongly on aniline–Sepharose, and was not eluted at $(NH_4)_2SO_4$ concentrations as low as 0.25M, but was only eluted by 0.25M-NaCl and 0.25M-Tris base. *p*-Aminobenzoic acid–Sepharose retained the enzyme only weakly, the enzyme being eluted in 1.0M- and 0.75M-$(NH_4)_2SO_4$.

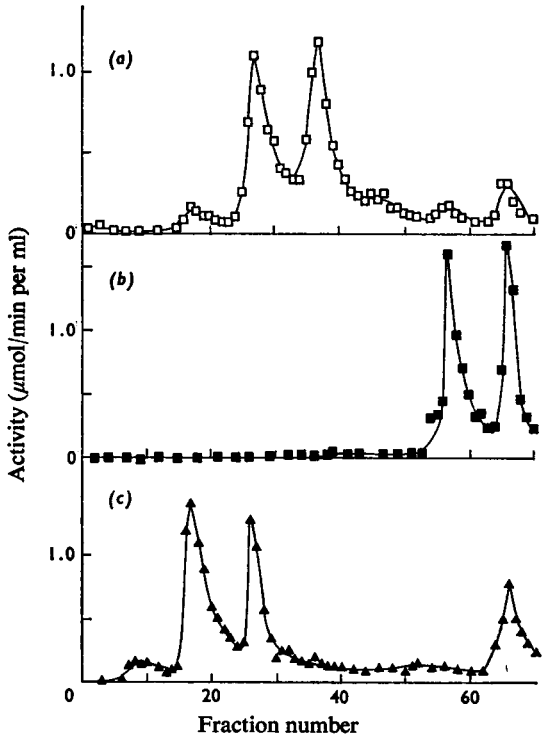


Fig. 3. *Elution profiles of human placental alkaline phosphatase on (a) D-phenylalanine–Sepharose, (b) aniline–Sepharose, (c) p-aminobenzoate–Sepharose*

The same enzyme sample as was used in Fig. 1 was applied to columns with the following dimensions: D-phenylalanine–Sepharose, 0.9cm×12.0cm; aniline–Sepharose, 0.9cm×10.5cm; *p*-aminobenzoate–Sepharose, 0.9cm×11.2cm. Conditions for binding and elution were the same as in Fig. 1.

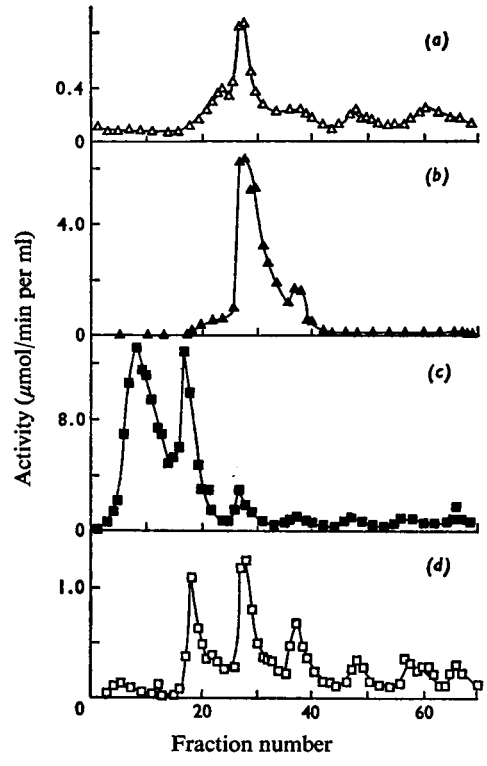


Fig. 4. *Elution profiles of alkaline phosphatases of (a) E. coli, (b) calf intestine, (c) chicken intestine and (d) pig intestine on L-phenylalanine–Sepharose*

Samples were applied in 1.25M-$(NH_4)_2SO_4$; total enzyme applied: *E. coli*, 10units; calf intestine, 30 units; chicken intestine, 80 units; pig intestine, 15units. Elution conditions were identical with those in Fig. 1.

Finally, alkaline phosphatases from different sources were tested for their retention on L-phenylalanine–Sepharose. As seen in Fig. 4, alkaline phosphatase from *E. coli*, which is not inhibited by L-phenylalanine, is eluted from L-phenylalanine–Sepharose at the same $(NH_4)_2SO_4$ concentration as calf intestinal alkaline phosphatase, which is inhibited by L-phenylalanine, and at a lower concentration of $(NH_4)_2SO_4$ than chicken intestinal and pig intestinal alkaline phosphatase, which are also inhibited by L-phenylalanine.

Purification of placental alkaline phosphatase

The starting material for this purification was material obtained from step 2 of the large-scale method for placental alkaline phosphatase, as published previously (Ghosh & Fishman, 1968). The dialysed butanol supernatant was adjusted to 90% saturation of $(NH_4)_2SO_4$, and the resulting precipitate was stored at −20°C until used. This material represented approximately a 10-fold purification of the enzyme, relative to homogenized placenta.

A batch (35g) of the $(NH_4)_2SO_4$ precipitate was dissolved in 100ml of cold (4°C) 0.05M-Tris–acetate buffer, pH8.0, and dialysed overnight against 4 litres of the same buffer at 4°C. The non-diffusible material, which contained particulate matter, was then sonicated with a Biosonik IV sonifier at high power for 15min in 3min bursts, with cooling to 5°C between bursts. (These conditions were found to increase maximally the specific activity of alkaline phosphatase.) Solid $(NH_4)_2SO_4$ was then added, gradually with stirring, to a final concentration of 1.25M. The solution was then centrifuged for 60min at 48000$g_{max.}$ in a Sorvall centrifuge.

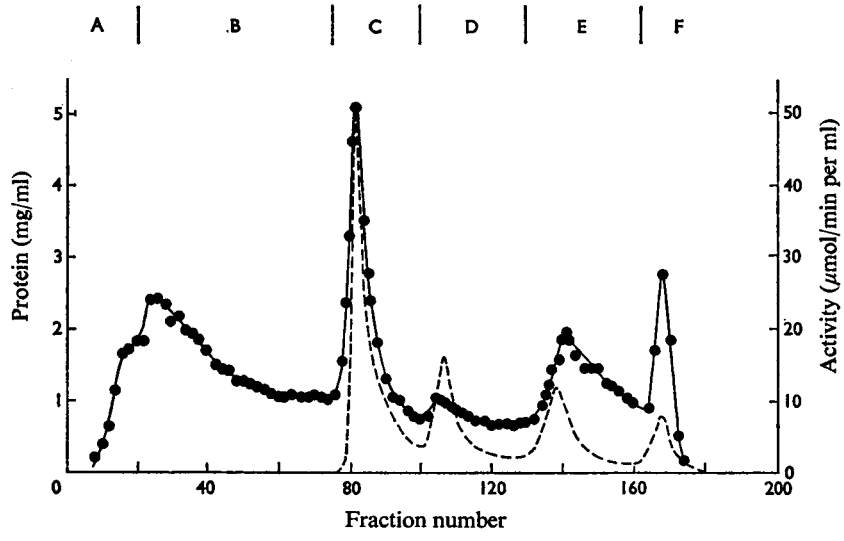


Fig. 5. *Elution profile of a large-scale preparation of human placental alkaline phosphatase on* L-*phenylalanine–Sepharose*

Enzyme preparation (200ml) containing 10.1mg of protein/ml with a specific enzyme activity of 4.1 was applied to a column (2.6cm×32cm) of L-phenylalanine–Sepharose in 0.05M-Tris–acetate buffer, pH8.0, containing 1.25M-$(NH_4)_2SO_4$ (A). The column was then eluted successively with: 500ml of 1.00M-$(NH_4)_2SO_4$–0.04M-Tris–acetate, pH8.0 (B); 300ml of 0.5M-$(NH_4)_2SO_4$–0.02M-Tris–acetate, pH8.0 (C); 300ml of 0.25M-$(NH_4)_2SO_4$–0.02M-Tris–acetate, pH8.0 (D); 300ml of 0.25M-NaCl–0.05M-Tris–acetate, pH8.0 (E); 300ml of 0.25M-Tris base, pH10.5 (F). The elution was performed at 4°C; the fraction size was 11ml. ●, Protein; ----, activity.

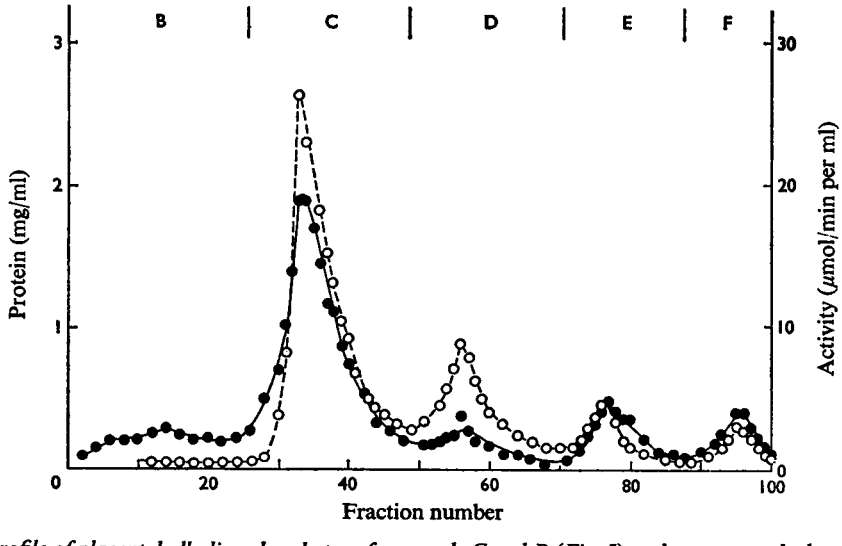


Fig. 6. *Elution profile of placental alkaline phosphatase from pools C and D (Fig. 5) rechromatographed on* L-*phenylalanine–Sepharose*

The pools were brought to 1.25M-$(NH_4)_2SO_4$ and 0.05M-Tris–acetate, pH8.0, by addition of 2.5M-$(NH_4)_2SO_4$ and 1.0M-Tris–acetate, pH8.0, and applied to L-phenylalanine–Sepharose. Column dimensions and eluting salt solutions were as in Fig. 5, except that 300ml of each salt solution was used. ●, Protein; ----, activity.

L-Phenylalanine–Sepharose chromatography

The supernatant from the centrifugation was applied to a column (2.6cm×32cm; bed volume 175ml) of L-phenylalanine–Sepharose equilibrated with 1.25M-$(NH_4)_2SO_4$–0.05M-Tris–acetate, pH8.0. Successive elutions with stepwise lower concentrations of $(NH_4)_2SO_4$ followed by NaCl and by Tris base resulted in the elution profile shown in Fig. 5. The enzyme is preferentially eluted in 0.50M- and 0.25M-$(NH_4)_2SO_4$, with some tailing into the fractions eluted later. The pool of 0.50M- and 0.25M-$(NH_4)_2SO_4$-eluted enzyme was then adjusted to 1.25M-$(NH_4)_2SO_4$ by addition of 2.5M-$(NH_4)_2SO_4$–0.10M-Tris–acetate, pH8.0, reapplied to the same column as was used in Fig. 5, and eluted with 300ml each of the same buffers as were used for the first column. The enzyme was again eluted in two main peaks at 0.50M- and 0.25M-$(NH_4)_2SO_4$, with some tailing into the 0.25M-NaCl and the 0.25M-Tris base eluates (Fig. 6).

A comparison of the total activity recovered in each peak of the two experiments is given in Table 1. Elution of placental alkaline phosphatase was comparable for the two columns, and almost all of the activity was accounted for in the pools from the two columns.

Table 1. *Elution of placental alkaline phosphatase from L-phenylalanine–Sepharose as a function of salt concentration and type*

For details see the text.

	Total activity (μmol/min)	
	Column 1 (Fig. 4)	Column 2 (Fig. 6)
Total enzyme applied	8102	4946
0.5M-$(NH_4)_2SO_4$ eluate	4008	2766
0.25M-$(NH_4)_2SO_4$ eluate	938	991
0.25M-NaCl eluate	822	417
0.25M-Tris base eluate	540	299
Total enzyme recovered	6414	4472
Recovery (%)	79.2	90.4

DEAE-Sephadex chromatography

Initial attempts to use DEAE-Sephadex chromatography for purification of placental alkaline phosphatase were unsatisfactory. The enzyme was not eluted as a discrete peak, and so only a slight purification could be obtained.

To circumvent this difficulty the observation (Fishman, 1974) that Triton X-100 could be used to enhance resolution in disc gel electrophoresis of alkaline phosphatase was applied to the DEAE-Sephadex chromatography of the enzyme. The pool of the enzyme from the 0.50M- and 0.25M-$(NH_4)_2SO_4$ eluates of the previous step was dialysed against two changes of 4 litres each of 0.02M-Tris–acetate, pH8.0, overnight, and was applied to a column (2.5cm×20cm) of DEAE-Sephadex A-25 equilibrated with

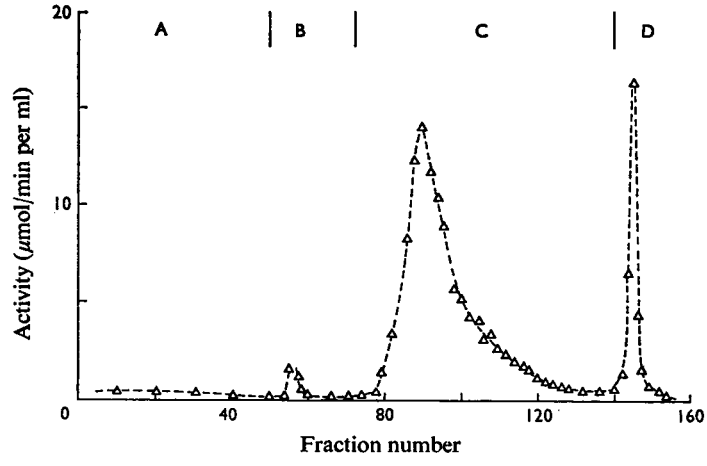


Fig. 7. *Elution profile of placental alkaline phosphatase from pools C and D (Fig. 6) rechromatographed on DEAE-Sephadex A-25*

The column dimensions were 2.5cm×20cm, and 15ml fractions were collected. The sample was applied in 0.02M-Tris–acetate, pH8.0 (A), washed with 0.05M-Tris–acetate–0.05% Triton X-100, pH8.0 (B), and the enzyme was then eluted with a gradient of 0–0.15M-sodium acetate in 0.05M-Tris–acetate–0.05% Triton X-100, pH8.0 (C). The column was then washed with 0.50M-sodium acetate–0.05M-Tris–acetate–0.5% Triton X-100, pH8.0 (D).

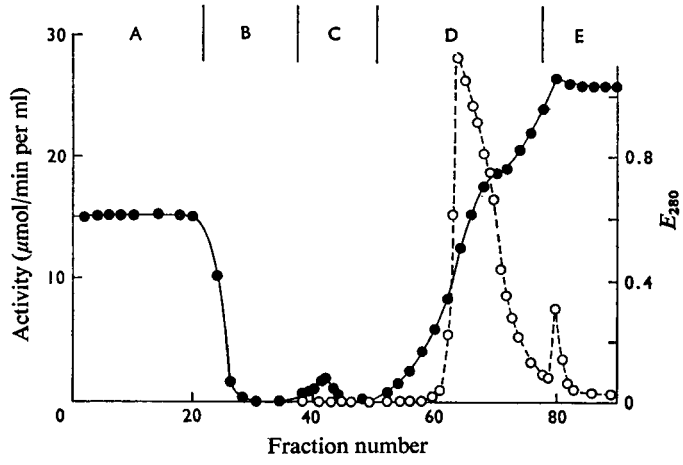


Fig. 8. *Elution profile of placental alkaline phosphatase obtained from the sodium acetate gradient in Fig. 7 rechromatographed on DEAE-Sephadex A-25*

The column dimensions were 1.5cm×15cm; 15ml fractions were collected. A sample was dialysed against a fivefold excess of deionized water and applied to the column, which was equilibrated with 0.05M-Tris–acetate, pH8.0 (A). The column was washed with 200ml of 0.05M-Tris–acetate, pH8.0 (B) and with 200ml of 0.1M-sodium acetate–0.05M-Tris–acetate, pH8.0 (C). The enzyme was then eluted with a gradient of 0–0.5% Triton X-100 in 0.1M-sodium acetate–0.05M-Tris–acetate, pH8.0 (D). Further washing with 0.15M-sodium acetate–0.05M-Tris–acetate–0.05% Triton X-100, pH8.0 (E) resulted in elution of only a slight amount of activity relative to the amount recovered in the Triton X-100 gradient in 0.1M-sodium acetate. ●, E_{280}; ○, activity.

0.02M-Tris–acetate, pH8.0. The column was then washed with 0.05M-Tris–acetate–0.05% Triton X-100 detergent, pH8.0, and eluted with a gradient of 0–0.15M-sodium acetate in the Triton X-100-containing buffer. As seen in Fig. 7, the enzyme was eluted at a sodium acetate concentration of 0.03–0.10M. (An E_{280} profile was not obtained for this column because of the high absorbance at 280nm of the Triton X-100 detergent.)

The peak tubes from the enzyme elution of the first DEAE-Sephadex column (tubes 82–100) were pooled and dialysed against 2 litres of deionized water overnight. They were then applied to a column (1.5cm×15cm) of DEAE-Sephadex A-25 equilibrated with 0.05M-Tris–acetate, pH8.0. A gradient of Triton X-100 of 0–0.05% in 0.1M-sodium acetate–0.05M-Tris–acetate, pH8.0, was then used to elute the enzyme. This was done to remove any proteins that were eluted with the enzyme in the sodium acetate gradient, and that were not affected by the Triton X-100 detergent. The elution profile for this column is shown in Fig. 8. Some protein was removed from this column in the 0.1M-sodium acetate wash, and the enzyme was eluted between 0.02 and 0.05% Triton X-100. Further elution with 0.15M-sodium acetate in buffer containing 0.05% Triton X-100 removed the small amount of enzyme remaining on the column.

As the final step in the purification, the peak tubes from the Triton X-100 gradient were dialysed overnight against 2 litres of deionized water, and applied to a column (0.9cm×10cm) of DEAE-Sephadex A-25 which was equilibrated with 0.05M-Tris–acetate, pH8.0. The column was then washed with 200ml of 0.05M-Tris–acetate, pH8.0, to remove the Triton X-100 from the enzyme, and with 15ml of 0.10M-sodium acetate–0.05M-Tris–acetate, pH8.0. The enzyme was then eluted with a gradient of 0.10–1.0M-sodium acetate, pH8.0. The enzyme was eluted with a peak at a sodium acetate concentration of 0.15–0.25M, as seen in Fig. 9.

Total recovery in the peak tubes from this column was only 440 units, or less than 20% of the total enzyme applied. Also, it was noted that considerable 'tailing' was apparent in the elution profile from this column. Elution with 1.0M-sodium acetate, after the column had been left overnight in the cold room (4°C), eluted another 170 units of activity. It appeared therefore that some enzyme was still bound to the column. The gel was therefore removed from the column and washed batchwise with 1.0M-sodium acetate–0.05M-Tris–acetate, pH8.0. As seen in Table 2, further amounts of enzyme were removed from the column by this procedure. The removal is therefore a time-dependent temperature-dependent process,

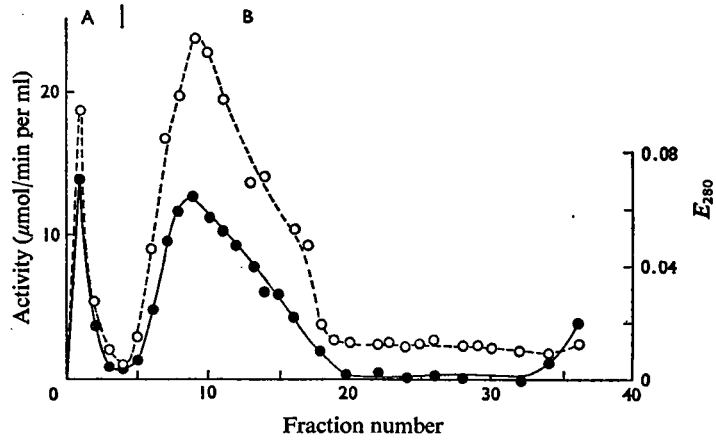


Fig. 9. *Elution profile of placental alkaline phosphatase obtained from the Triton X-100 gradient of Fig. 8 on a column of DEAE-Sephadex A-25*

The column dimensions were 0.9cm×10cm; 3ml fractions were collected. The pool of enzyme was dialysed against a fivefold excess of deionized water and applied to the column, which was equilibrated with 0.05M-Tris–acetate, pH8.0. The column was washed with 200ml of 0.05M-Tris–acetate, pH8.0, and with 15ml of 0.1M-sodium acetate–0.05M-Tris–acetate, pH8.0 (A). The enzyme was then eluted with a gradient of 0.1–1.0M-sodium acetate in 0.05M-Tris–acetate, pH8.0 (B). ●, E_{280}; ○, activity.

Table 2. *Batchwise elution of placental alkaline phosphatase from DEAE-Sephadex from which enzyme had been eluted by using a gradient of 0.10–1.0M-sodium acetate*

The gel was removed from the column after elution of the enzyme and washed under various conditions, as indicated.

Step	Volume (ml)	Buffer	Total activity (μmol/min)
1	26	1M-Sodium acetate–0.05M-Tris–acetate	381
2	50	1M-Sodium acetate–0.05M-Tris–acetate	318
3	54	1M-Sodium acetate–0.05M-Tris–acetate	82
4	46	1M-Sodium acetate–0.05M-Tris–acetate	32
5	46	1M-Sodium acetate–0.05M-Tris–acetate Suspension incubated at 37°C for 30min	27
6	49	1M-Sodium acetate–0.05M-Tris–acetate Suspension incubated at 37°C for 60min	78
7	51	1M-Sodium acetate–0.05M-Tris–acetate	82
8	54	0.1M-Sodium acetate–0.05M-Tris–acetate, 0.05% Triton X-100	180

which can be facilitated by use of Triton X-100 detergent.

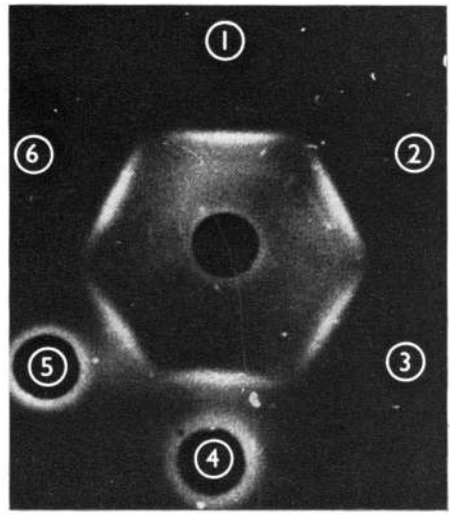
Pools from the elution of the enzyme were freeze-dried and dialysed overnight against 4 litres of de-ionized water, freeze-dried again, dialysed overnight against 0.01M-Tris–acetate–0.1mM-zinc acetate–20% glycerol, and stored in this buffer at 0°C.

A summary of the purification of the enzyme is presented in Table 3. The enzyme has been purified 840-fold relative to homogenized placenta. A final recovery of 22% of the activity applied to the first L-phenylalanine–Sepharose column was obtained, and a final specific activity of 160μmol/min per mg of protein, measured under the conditions of enzyme assay at pH9.8, and with 18mM-phenyl phosphate as substrate.

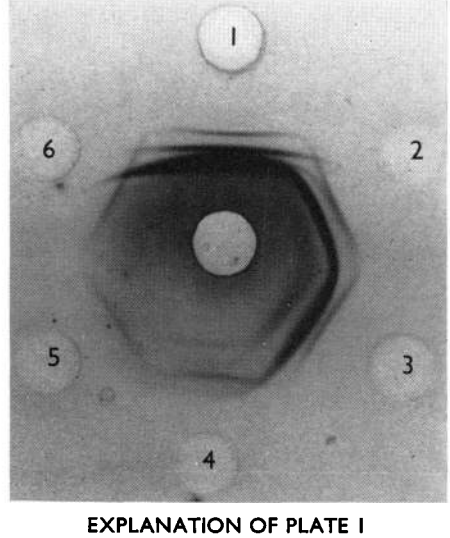
Criteria of purity

Immunological. The result for the diffusion of the pools from the above enzyme preparation against rabbit antibody prepared from starting material is shown in Plate 1. The single precipitin line corresponded to the position for the enzyme activity, as

(*a*)



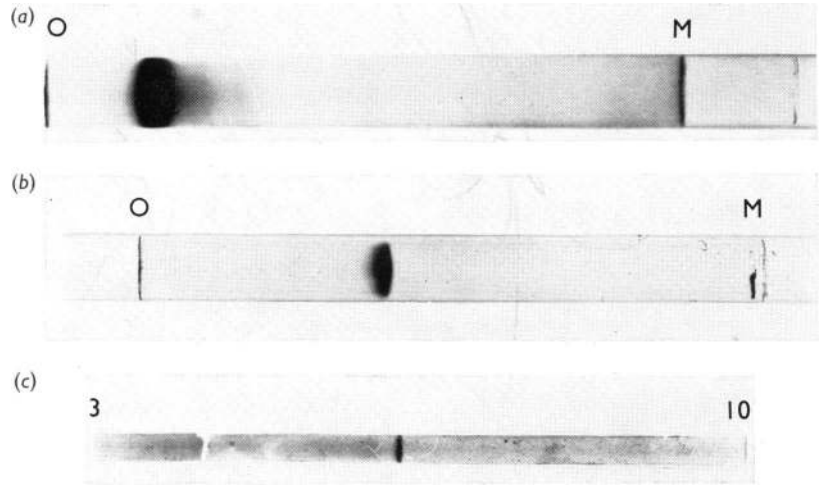
(*b*)



EXPLANATION OF PLATE 1

Ouchterlony double-diffusion of antibody prepared from the butanol-treated dialysed placental homogenate against the pools from the various steps in the purification of placental alkaline phosphatase

(*a*) Gel in which alkaline phosphatase was detected by naphthol AS-MX phosphate hydrolysis and photography under u.v. light; (*b*) protein stain of the same plate. (For details, see the Experimental Procedures section.) Wells contained (1) 0.80unit of activity from the sonicated 1.25M-$(NH_4)_2SO_4$ supernatant; (2) 0.66unit of activity from the first L-phenylalanine–Sepharose pool; (3) 0.51unit of activity from the second L-phenylalanine–Sepharose pool; (4) 0.44unit of activity from the first DEAE-Sephadex (sodium acetate gradient) pool; (5) 0.48unit of activity from the peak of the second DEAE-Sephadex column; (6) 0.40unit of activity from the peak of the third DEAE-Sephadex column.



EXPLANATION OF PLATE 2

Enzyme obtained from the peak elution of the sodium acetate gradient of the third DEAE-Sephadex column (Fig. 9) was analysed as follows: (*a*) Polyacrylamide-gel electrophoresis of 25 μg of enzyme stained for protein. O indicates the origin and M the location of the marker dye. (*b*) Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of 10 μg of enzyme stained for protein. O indicates the origin and M the location of the marker dye. (*c*) Isoelectric focusing of 5 μg of enzyme on polyacrylamide gels having a gradient of pH from 3 (left) to 10 (right) stained for protein. Gel dimensions 0.3 × 7.2 cm.

Table 3. *Purification procedure for human placental alkaline phosphatase*

Step	Protein (mg)	Activity (μmol/min)	Specific activity (μmol/min per mg of protein)	Purification	Recovery total (%)
Tris homogenate	21326	3924	0.184	1	100*
Dialysed $(NH_4)_2SO_4$ precipitate	2500	3375	1.35	7.3	86*
Sonicated 1.25M-$(NH_4)_2SO_4$ supernatant	2020	8102	4.05	23.9	206
First L-phenylalanine–Sepharose column	336	4946	14.7	79.9	126
Second L-phenylalanine–Sepharose column	227	3757	16.5	89.7	96
DEAE-Sephadex column; sodium acetate gradient (+Triton X-100)	—	3440	—	—	88
DEAE-Sephadex column; Triton X-100 gradient	—	2977	—	—	76
Final DEAE-Sephadex column					
Gradient	3.25	438	135	734	11.2
First sodium acetate wash	1.02	170	167	908	4.3
Successive washings	7.28	1180	162	880	30.1
Total	11.55	1788	155	842	45.6

* Taken from Ghosh & Fishman (1968).

seen in the photograph of fluorescence from the hydrolysis of naphthol AS-MX phosphate (*a*). The enzyme prepared by the above procedure is therefore antigenically identical with the starting material, and is apparently homogeneous, as indicated by a single precipitin line in the pools from the second and third DEAE-Sephadex columns.

Electrophoresis. Results for polyacrylamide-disc-gel electrophoresis (*a*), sodium dodecyl sulphate–polyacrylamide-gel electrophoresis (*b*) and gel isoelectric focusing (*c*), stained for protein, are shown in Plate 2. The enzyme activity was found to be located at the same position as the stainable protein in the case of the disc gel electrophoresis and isoelectric-focusing gels. The p*I* of the enzyme was 6.9–7.0 by this method.

Determination of subunit molecular weight. Five determinations of the relative mobility of placental alkaline phosphatase on sodium dodecyl sulphate–polyacrylamide gels by the method of Fairbanks *et al.* (1971), and three determinations each of the standards bovine serum albumin (mol.wt. 66500), aldolase (mol.wt. 39500), ovalbumin (mol.wt. 43500), chymotrypsinogen A (mol.wt. 23200) and ribonuclease A (mol.wt. 13700) yielded a subunit molecular weight of 70000 for human placental alkaline phosphatase. This corresponds to the molecular weight reported for the A form of human placental alkaline phosphatase (Ghosh & Fishman, 1968).

Discussion

The retention of alkaline phosphatases, specifically human placental alkaline phosphatase, on Sepharose derivatives at high concentrations of $(NH_4)_2SO_4$ did not appear to be related to a specific 'affinity chromatography' of placental alkaline phosphatase. Thus the retention of placental alkaline phosphatase on D-phenylalanine–, L-leucine–, aniline– and *p*-aminobenzoic acid–Sepharoses, in particular, does not correspond to any known function for these ligands. Also, the retention of *E. coli* alkaline phosphatase on L-phenylalanine–Sepharose clearly demonstrates that an enzyme that is not inhibited by L-phenylalanine can be retained on this column.

The binding of phosphatase to the Sepharose derivatives studied in this work was unrelated to the L-phenylalanine-binding site of placental alkaline phosphatase. Yet there was evidence of interaction with enzyme of the phenyl group present in various modified Sepharoses examined. Accordingly, this phenomenon fits to some extent the 'hydrophobic-affinity' effect in protein purification (Hofstee, 1973; Shaltiel & Er-El, 1973). With regard to the latter effect, binding to aniline–Sepharose could be expected to be stronger than to L-phenylalanine–Sepharose or to *p*-aminobenzoic acid–Sepharose, since aniline–Sepharose does not contain a polar carboxyl group in the vicinity of the phenyl group, which could limit hydrophobic interaction. This was the case.

Studies on the effects of salts in other systems lend support to this interpretation. In particular, the 'salting-out' of proteins (Dixon & Webb, 1961), and of small organic molecules and gases (Long & McDevitt, 1952), and the effects of salts on membrane-bound protein complexes (Hatefi & Hanstein, 1969) have been interpreted as being due to an effect of ions on water structure that could stabilize (e.g. sulphate) or decrease (e.g. iodide) hydrophobic interactions (Hatefi & Hanstein, 1969; Hanstein *et al.*, 1971; von Hippel & Schleich, 1969). This interpretation of the effect of sulphate ions on interactions in aqueous solution correlates very well with the interpretation of the sulphate-mediated binding to Sepharose deri-

vatives studied above as being due to hydrophobic interaction.

The effect of Triton X-100 on chromatography on DEAE-Sephadex appears to be twofold. First, it changes the ionic strength at which the enzyme is eluted, and secondly, it affects the non-ionic binding of the enzyme to DEAE-Sephadex. We have further found that the isoelectric pH of the enzyme is altered in Triton X-100 (D. Angellis & W. H. Fishman, unpublished work), and that the migration of the enzyme on polyacrylamide gels is enhanced (Fishman, 1974).

Since the discovery of the Regan isoenzyme, a carcinoma-associated alkaline phosphatase phenotype that is apparently identical with placental alkaline phosphatase (Fishman *et al.*, 1968, 1971), a simplified purification method that could be used to purify and characterize placental and Regan isoenzymes in higher yield than was previously obtainable (Ghosh & Fishman, 1968) has been a goal of our laboratory. The method outlined above can be used for this purpose.

This research has been supported in part by Grants-in-aid from the National Cancer Institute, National Institutes of Health (CA-12924; CA-13332; K6-CA-18,453) and from the John A Hartford Foundation, New York.

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