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DESIGN AND APPLICATIONS OF BIOMIMETIC ANTHRAQUINONE DYES

PURIFICATION OF CALF INTESTINAL ALKALINE PHOSPHATASE WITH IMMOBILISED TERMINAL RING ANALOGUES OF C.I. REACTIVE BLUE 2

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SUMMARY

A 330-fold one-step purification of alkaline phosphatase from a crude calf intestinal extract has been achieved using specific elution with inorganic phosphate (5 m*M*) from a purpose designed adsorbent comprising a terminal ring phosphonate analogue of C.I. Reactive Blue 2 coupled to Sepharose CL-6B-200. The resulting alkaline phosphatase preparation displayed a specific activity in excess of 1000 U/mg and was of equivalent purity to commercial "high purity" preparations as deduced by sodium dodecyl sulphate polyacrylamide gel electrophoresis and specific activity comparisons.

INTRODUCTION

Highly purified alkaline phosphatase [orthophosphoric monoester phosphohydrolase (alkaline optimum), E.C. 3.1.3.1] from calf intestinal mucosa is widely used in molecular biology and immunodiagnostics. Traditionally, the enzyme has been purified by a lengthy, multi-step procedure employing conventional chromatographic techniques[1,2]. However the reported yields are low and the final preparations are invariably contaminated with co-purifying enzymes such as phosphodiesterase (oligonucleate 5'-nucleotidohydrolase, E.C. 3.1.4.1)[3]. More recently, however, affinity chromatography has been successfully exploited for the purification of alkaline phosphatase from calf intestine[1,2] and other sources[4] using immobilised competitive inhibitors such as phosphonic and arsanilic acids as affinity ligands. Unfortunately, these derivatives tend to be difficult and expensive to synthesise, require relatively hazardous activation conditions to couple to the solid support and yield adsorbents with ill defined ligand concentrations and which display limited chemical and biological stability. These problems may well be ameliorated by the introduction of triazine dyes as affinity ligands[5]. For example, a number of commercially available

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triazine dyes have been screened for their ability to purify alkaline phosphatase from crude intestinal preparations[6]. Most of the immobilised triazine dyes bound the enzyme to some extent and a similarity between the sulphonated aromatic dyes and other alkaline phosphatase affinity ligands was noted. The use of selected dyes, when combined with specific elution, resulted in high purification factors. Immobilised dyes have also been used as components in a multiple-step calf intestine alkaline phosphatase purification procedure[7] and for the purification of alkaline phosphatases from other sources[8–10].

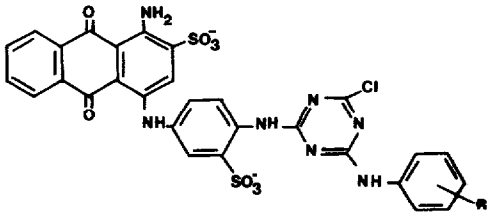
Specifically designed biomimetic dyes which display higher affinity for horse liver alcohol dehydrogenase than conventional commercially available dyes have recently been synthesised and mark the onset of a new era in the use of triazine dyes for protein purification[11,12]. This paper reports a similar rational approach to synthesise anthraquinone dyes based on C.I. Reactive Blue 2 bearing phosphonate and other terminal ring substituents for the purification of calf intestinal mucosa alkaline phosphatase.

EXPERIMENTAL

Materials

The commercial triazine dyes (Procion™ H and MX series) and the terminal ring bases, aniline, *p*-aminobenzoic acid, *m*- and *p*-aminobenzenesulphonic acids, *p*-aminobenzyl phosphonic acid and *m*-aminobenzeneboronic acid were a generous and much appreciated gift from Dr. C. V. Stead, ICI Organics Division, Manchester, U.K. The terminal ring analogues of C.I. Reactive Blue 2 (Fig. 1, I–V) were synthesised according to a previously published procedure[13].

Sepharose CL-6B-200, bovine serum albumin (fraction V powder, 98–99% albumin), calf intestinal alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), E.C. 3.1.3.1; Type I, 1–3 U/mg solid] and all other crude alkaline phosphatase preparations, phosphodiesterase I (oligonucleate 5′-nucleotide hydrolase, E.C. 3.1.4.1, bovine intestinal mucosa, 0.5–1 U/mg protein), bis(*p*-nitrophenyl) phosphate, α-naphthyl phosphate and 4-phenyl phosphate were all obtained from Sigma (London), Poole, U.K. High-purity alkaline phosphatase (enzyme label



	TERMINAL RING ANALOGUE	R
I	aniline	-H
II	p-aminobenzoic acid	-COOH
III	m/p-aminobenzenesulphonic acid	$-SO_3H$
IV	p-aminobenzyl phosphonic acid	$-CH_2PO_3H_2$
V	m-aminobenzeneboronic acid	$-B(OH)_2$

Fig. 1. Structures of the terminal ring analogues of C.I. Reactive Blue 2.

for enzyme immunoassay-grade I) was obtained from Boehringer Mannheim, Lewes, U.K. and from Biozyme Labs., Gwent, U.K. (ALP I–10 G). All other chemicals were of Analar grade.

Assay of enzyme activities and protein

Alkaline phosphatase and phophodiesterase activities were assayed by following the production of *p*-nitrophenolate anion at 405 nm. For alkaline phosphatase the total assay volume of 1 ml contained: glycine–NaOH buffer, pH 10.4 (0.1 mmol), *p*-nitrophenyl phosphate (6 μmol), $MgCl_2$ (1 μmol) and $ZnCl_2$ (1 μmol). The assay mixture for the phosphodiesterase contained, in a total volume of 1 ml: Tricine–NaOH buffer, pH 8.8 (60 μmol) and bis(*p*-nitrophenyl) phosphate (6 μmol). The molar extinction coefficient of the *p*-nitrophenolate anion was taken as 18 500 l mol^{-1} cm^{-1} at 405 nm. One unit of enzyme activity is defined as that amount of enzyme which catalyses the formation of 1 μmol *p*-nitrophenolate anion per min at 37°C.

Protein concentrations were determined by measurement of the absorbance at 280 nm using a mass extinction coefficient of 1.0 absorbance unit per mg ml^{-1} of protein for alkaline phosphatase[14].

Immobilisation of triazine dyes

Sepharose CL-6B-200 was exhaustively washed with distilled water, sucked moist on a sintered funnel and divided into 5-g portions. The commercial dye powders were washed with diethyl ether and samples (100 mg) dissolved in water (22.5 ml) and added to the moist gel (5 g). The suspension was incubated for 5 min at 20°C prior to the addition of 20% (w/v) NaCl solution (2.5 ml). The suspension was gently agitated for 30 min at 55°C whence solid Na_2CO_3 (250 mg) was added to a final concentration of 1% (w/v). Dichlorotriazinyl dyes (Procion MX series) and monochlorotriazinyl dyes (Procion H series and C.I. Reactive Blue 2 analogues) were incubated for 3 h and 10–50 h respectively in a rotary incubator at 55°C. The dyed gels were thoroughly washed with distilled water with the dichlorotriazinyl dyed gels further tumbled for 2–3 h with 1 *M* NH_4Cl (pH 8.7) in order to aminate unreacted chlorines. All dyed gels were finally washed with 1 *M* NaCl–25% (v/v) ethanol (100 ml), distilled water (200 ml) and equilibration buffer (200 ml). The dyed Sepharose adsorbents were stored as moist gels in distilled water containing 0.01% (w/v) thimerosal at 4°C.

Determination of immobilised ligand concentration

The immobilised dye concentrations were determined by hydrolysis of moist gel (0.2 g) in a known volume of 50% (v/v) glacial acetic acid at 95–100°C for 30 min. The absorbance of the resulting solution was measured at the λ_{max} of each dye tested and the concentration calculated as μmol dye/g moist weight gel. All dyes were immobilised at a ligand concentration of 1–3 μmol dye/g moist weight Sepharose.

Preparation of crude enzyme solutions

All crude phosphatase-containing solutions (*ca.* 6000 U, 5 g solid, 25 ml) were dialysed overnight at 4°C against equilibration buffer (2.5 l) before use.

Screening procedure for immobilised dye adsorbents for alkaline phosphatase and protein binding

Enzyme and protein binding was assessed using immobilised dye adsorbents (0.5 g moist weight gel) suspended in a small test tube containing crude enzyme preparation (25 U alkaline phosphatase) in 20 m*M* HEPES–NaOH buffer pH 7.5 (2 ml). After thorough mixing, the tubes were incubated for 30 min at 25°C whence after gravity sedimentation of the gels, the supernatants were assayed for protein and enzyme activity.

The gel phase was washed with equilibration buffer (2 × 4 ml), bound protein desorbed with 0.75 *M* KCl and the supernatant fraction assayed again for protein and alkaline phosphatase activity. Unmodified Sepharose CL-6B-200 was used as a control.

Screening of the C.I. Reactive Blue 2 analogues for their ability to bind alkaline phosphatase and bovine serum albumin was performed as the above using "Good" buffers (10 m*M*) at their respective pK_a values in the pH range 6.1–10.4.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli[15] using a Bio-Rad Mini-Protean II dual slab cell. Gels were stained for 30 min with 0.1% (w/v) Coomassie Blue R-250 in methanol–glacial acetic acid–water (40:10:50, v/v/v) then destained for 1 h in the same solvent.

RESULTS

Preliminary screen for alkaline phosphatase binding to immobilised triazine dyes

The results of screening a number of commercially available Procion H and MX dyes immobilised to agarose for their ability to bind and purify calf intestinal alkaline phosphatase are summarised in Table I. The screen was designed to encompass the principal categories of dye chromophore (anthraquinone, azo, phthalocyanine and metal complex) exploited in reactive dyes[5] to determine if any one dye category favoured binding of the enzyme. In agreement with the general finding of our other workers[6], all of the immobilised dye adsorbents bound the enzyme from a crude calf intestinal mucosa preparation at pH 7.5. However, in this study the dyes were immobilised at comparable ligand concentrations (1–3 μmol dye/g moist weight gel) thus allowing more precise comparisons of enzyme binding capacities on a U/μmol dye basis. Immobilisation of dyes at similar substitution levels is often overlooked in such studies but is important since high ligand concentrations may increase non-specific interactions[5]. Highest capacities for enzyme binding were displayed by those dyes bearing a significant number of anionic groups, such as immobilised Procion Green H-4G, Procion Green HE-4BD, Procion Turquoise H-A and Procion Blue MX-4GD. However, the most effective for enzyme purification was the azo dye, Procion Red P-3BN as it consistently achieved the highest purification factor, was easily coupled to the matrix and, unlike the large phthalocyanine dyes, was not prone to ligand leakage from the matrix. Interestingly, this dye was unique amongst those tested in possessing both amino phenyl sulphonic and amino benzoic acid groups. Subsequent studies using the Procion Red P-3BN adsorbent in conjunction with 20 m*M* orthophosphate as specific eluent resulted in an alkaline phosphatase preparation of specific activity

TABLE I

PRELIMINARY SCREEN FOR CALF INTESTINE ALKALINE PHOSPHATASE BINDING TO COMMERCIAL TRIAZINE DYES

Dialysed crude alkaline phosphatase preparation (25 U in 2 ml of 20 m*M* HEPES–NaOH, pH 7.5) was added to a small test tube containing immobilised dye adsorbent (0.5 g moist weight gel). After thorough mixing the tubes were incubated for 30 min at 25°C whence after gravity sedimentation of the gels, the supernatants were assayed for protein and enzyme activity. After removal of the supernatant, the gel phase was washed with equilibration buffer (2 × 4 ml), bound protein desorbed by the addition of 0.75 *M* KCl (2 ml) and the supernatant fraction reassayed for protein and enzyme activity. Unmodified Sepharose was used as a control, protein and enzyme recoveries were >90% and the experiment was performed in duplicate. The specific activity of the crude preparation was 3 U/mg protein.

Immobilised dye (Procion)	*Immobilised ligand concentration (μmol/g moist gel)*	*Binding capacity (U/μmole dye)*	*Specific activity (U/mg protein)*
Control	0.0	–	–
Reactive Blue 2	2.2	6.2	22.6
Blue MX-4GD	2.6	9.8	39.4
Blue HE-RD	2.6	9.6	25.9
Red H8-BN	2.3	5.2	25.6
Red H-3B	2.0	0.7	15.2
Red HE-3B	2.7	6.8	29.4
Red P-3BN	3.0	8.3	51.3
Red HE-7B	3.0	9.7	33.2
Yellow MX-R	3.0	0.6	8.5
Yellow H-A	1.8	1.0	8.2
Green HE-4BD	1.2	23.3	33.2
Green H-4G	1.0	42.3	33.8
Brown H-2G	3.0	3.4	29.7
Brown MX-5BR	2.5	2.0	27.9
Turquoise H-A	1.0	10.8	24.0
Orange MX-G	1.2	0.9	6.2
Scarlet MX-G	2.7	1.4	14.6

180 U/mg and representing a 60-fold purification. Since commercial "high-purity" alkaline phosphatase preparations have specific activities in the range 850–1250 U/mg under these conditions, the preparation reported here was only partially purified and comparable to that obtained by other workers using other commercial dyes for alkaline phosphatase purification[6].

Design, immobilisation and application of specific dyes targeted at alkaline phosphatase

The relative lack of specificity of commercial dyes in their interaction with alkaline phosphatase and the apparent relationship with the number of anionic groups substituted on the dye chromophore suggest that these groups may act as phosphate analogues. Thus, since carboxylate[16], phosphonate[3] and borate[16] are known to be potent competitive inhibitors of alkaline phosphatase and the enzyme generally favours aromatic rather than aliphatic phosphate esters, substitution of these groups onto aromatic dye chromophores would be expected to yield more effective adsorbents. Fig. 1 shows the structure of a series of terminal ring analogues of the anthraquinone dye, C.I. Reactive Blue 2, which were synthesised, immobilised and

TABLE II

PURIFICATION OF CALF INTESTINAL ALKALINE PHOSPHATASE ON IMMOBILISED TERMINAL RING ANALOGUES OF C.I. REACTIVE BLUE 2

Enzyme and protein binding was assessed over the pH range 6.1–10.4 using immobilised dye adsorbents (0.5 g moist weight gel) suspended in a small test tube containing crude enzyme (25 U alkaline phosphatase) in 20 m*M* "Goods" buffer (2 ml). After thorough mixing, the tubes were incubated for 30 min at 25°C, the gels allowed to sediment under gravity and the supernatants assayed for protein and enzyme activity. The gels were washed with equilibration buffer (2 × 4 ml), bound protein desorbed with 0.75 *M* KCl and the supernatants assayed for protein and enzyme activity. Total protein and enzyme recoveries were >90% and the experiment was performed in duplicate.

pH	*Terminal ring analogue*[a]				
	I	*II*	*III*	*IV*	*V*
6.1	5.6[b] (14)[c]	6.1 (19)	6.2 (17)	6.2 (17)	2.1 (11)
7.2	2.9 (30)	5.8 (72)	4.5 (52)	5.9 (83)	1.6 (36)
8.1	1.0 (20)	4.2 (105)	2.4 (62)	4.2 (124)	1.1 (44)
9.3	0.09 (4)	0.13 (5)	0.14 (19)	3.4 (200)	0.3 (33)
10.4	0	0	0	0	0

[a] See Fig. 1 for structures.
[b] Alkaline phosphate bound (U/μmol dye).
[c] Specific activity of eluted enzyme (U/mg protein).

tested for their ability to bind alkaline phosphatase from a crude calf intestinal mucosa extract over the pH range 6.1–10.4 (Table II). These analogues bear terminal rings comprising aniline (I), *p*-aminobenzoic acid (II), *m*-, *p*-aminobenzenesulphonic acid (C.I. Reactive Blue 2) (III), *p*-aminobenzyl phosphonic acid (IV) and *m*-aminobenzeneboronic acid (V). In each case, the capacity of the adsorbents (U enzyme bound/μmol immobilised dye) decreased with increasing pH, whilst the specific activity of the protein eluted with 0.75 *M* KCl increased with increasing pH up to a critical value after which the binding ability sharply declined. This critical value was about a pH unit higher for the phosphonate ligand. As anticipated, the phosphonate ligand was the most effective in yielding enzyme of high specific activity, with the carboxylate, sulphonate, borate and aniline derivatives decreasingly effective. Identification of the phosphonate analogue as an effective affinity ligand was probably attributable to the fact that the methyl phosphonate moiety has approximately the same overall dimensions and charge as the phosphate ester[17]. The excellent performance of the carboxylic acid analogue as an alkaline phosphatase ligand may account for dye Procion Red P-3BN being the most effective of the commercial dyes.

The terminal ring analogues of C.I. Reactive Blue 2 were also screened for their ability to bind bovine serum albumin, a major contaminant of the crude calf intestinal preparation (Table III). Bovine serum albumin bound most effectively to the aniline terminal ring analogue (I) and the monovalent sulphonate (III) and carboxylate (II) species. In sharp contrast to alkaline phosphatase binding, the phosphonate analogue (IV) displayed the lowest affinity for bovine serum albumin, thus, making it an ideal candidate ligand for the purification of calf intestinal alkaline phosphatase.

For these enzyme and protein binding studies, the Good buffers were used at their respective pK_a values in order to maintain a constant ionic environment over the pH range investigated as the buffers would be equally dissociated.

TABLE III

THE BINDING OF BOVINE SERUM ALBUMIN TO IMMOBILISED TERMINAL RING ANALOGUES OF C.I. REACTIVE BLUE 2

Bovine serum albumin binding was assessed over the pH range 6.1–10.4 using immobilised dye adsorbents (0.5 g moist weight gel) suspended in a small test tube containing protein solution (2 mg bovine serum albumin in 2 ml of 20 m*M* Goods buffer). After thorough mixing the tubes were incubated for 30 min at 25°C, the gels allowed to sediment under gravity and the supernatants assayed for protein. The gels were washed with equilibration buffer (2 × 4 ml), bound protein desorbed with 0.75 *M* KCl and the supernatants reassayed for protein. The experiment was performed in duplicate and protein recoveries were in the range 95–110%.

pH	*Terminal ring analogue*[a]				
	I	*II*	*III*	*IV*	*V*
6.1	1.9[b]	1.2	1.2	0.8	1.1
7.2	1.4	1.0	1.1	0.7	0.9
7.5	1.2	0.8	1.0	0.6	0.7
8.1	0.5	0.3	0.4	0.2	0.3
9.3	0	0	0	0	0
10.4	0	0	0	0	0

[a] See Fig. 1 for structures.
[b] Bovine serum albumin bound (mg BSA/μmol dye).

Determination of the capacity of the immobilised phosphonate analogue (IV) of C.I. Reactive Blue 2 for alkaline phosphatase

The interaction between alkaline phosphatase and the agarose-immobilised phosphonate analogue (IV) was strongly dependent on buffer concentration. For example, at a fixed pH value of 8.5 using Tricine–NaOH buffer, 85% of the total activity (140 U; 4 ml) bound when applied to a small column (1 g moist weight gel) of immobilised phosphonate ligand in 10 m*M* buffer, 70% bound in 40 m*M* buffer and only 35% bound when 100 m*M* buffer was used. A pH of 8.5 was chosen as optimum since at this value the phosphonate ligand is very selective for alkaline phosphatase whilst retaining a high binding capacity for the enzyme (Table I). Frontal analysis (Fig. 2) showed binding to be a complex process[18]; in the early phase, the majority of the total protein, but only 50% of the total enzyme activity is adsorbed. In the latter phase, a small amount of additional protein binds, representing the remaining enzyme activity. Alkaline phosphatase represents only 0.3% of the total protein in the crude preparation and the first phase is thought to reflect rapid saturation of potential binding sites on the adsorbent with alkaline phosphatase and contaminating proteins. The second phase may represent a slower time-dependent displacement by alkaline phosphatase of proteins possessing a lower affinity for the immobilised dye.

The volume of crude enzyme preparation necessary to achieve adsorbent saturation was highly dependent on column flow-rate. For example, at a flow-rate of 10 ml/h the volume required for saturation with alkaline phosphatase activity was 18 ml while at 20 ml/h the volume was almost doubled to 35 ml. This finding suggests that a competitive displacement effect may account for the complex binding process discussed above.

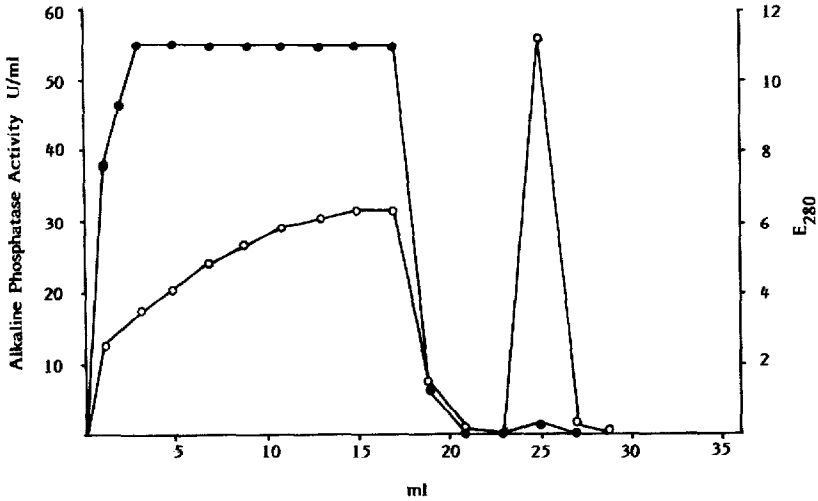


Fig. 2. Determination of maximum binding capacity of the terminal ring phosphonate analogue of C.I. Reactive Blue 2 (IV) when bound to Sepharose CL-6B-200 for alkaline phosphatase by frontal analysis. A crude calf intestinal alkaline phosphatase preparation (30 U/ml in 10 m*M* Tricine–NaOH buffer, pH 8.5) was applied to a glass column (10 cm × 0.5 cm I.D.) packed with phosphonate gel (0.1 g moist weight; 2.6 μmol dye/g moist weight gel) equilibrated with 10 m*M* Tricine–NaOH buffer pH 8.5 at 4°C at a flow-rate of 10 ml/h. Application was continued until outlet and inlet streams contained identical concentrations of enzyme and protein. The column was washed with the irrigating buffer and bound protein eluted with 0.75 *M* KCl. Fractions (1 ml) were assayed for alkaline phosphatase activity (○) and protein (E_{280}, ●).

A similar frontal chromatogram was observed for adsorption of alkaline phosphatase to immobilised Procion Red P-3BN, although at comparable dye substitution levels (2.6 μmol dye/g moist weight gel) the binding capacity for the immobilised Procion Red P-3BN (751 U/g moist weight gel; 289 U/μmol dye) was markedly lower than that for the immobilised phosphonate analogue (1147 U/g moist weight gel; 441 U/μmol dye). Only 5% of the bound protein appeared to be alkaline phosphatase in the case of immobilised Procion Red P-3BN, compared to 22% in the case of the phosphonate analogue; consequently, the specific activity of the enzyme eluted with 0.75 *M* KCl was 55 U/mg and 195 U/mg respectively. This demonstrates the increased selectivity of the phosphonate ligand over the commercial dyes. SDS-PAGE showed that the contaminating proteins included bovine serum albumin and phosphodiesterase.

Specific elution techniques

Desorption of alkaline phosphatase from the immobilised phosphonate analogue with steps (0.75 *M*) or linear (0–1 *M*) gradients of salt (KCl) resulted in co-elution of contaminating proteins with the enzyme activity. Table IV shows that the use of specific eluents such as phenyl phosphate, α-naphthyl phosphate and inorganic phosphate greatly improved the specific activity of the eluted protein. Specific elution with organic phosphates resulted in sharper elution of alkaline phosphatase although the degree of purification was consistently lower than that obtained with inorganic phosphate. Furthermore, SDS-PAGE showed that preparations eluted with organic

TABLE IV

SPECIFIC ELUTION OF ALKALINE PHOSPHATASE FROM IMMOBILISED PHOSPHONATE ANALOGUE OF C.I. REACTIVE BLUE 2

Total protein and enzyme recoveries were >90%.

Eluent	*Specific activity of eluted enzyme (U/mg protein)*	*Purification (fold)*[a]
0.75 *M* KCl	195	65
5 m*M* α-naphthyl phosphate	487	162
5 m*M* phenylphosphate	520	173
5 m*M* KH_2PO_4	716	238

[a] Crude alkaline phosphatase has a specific activity of 3.0 U/mg protein.

phosphates were highly contaminated with phosphodiesterase, whilst preparations eluted with inorganic phosphate were essentially free of this contaminant. Triazine dyes are competitive inhibitors of bovine phosphodiesterase and have been exploited for its purification[19,20]. Thus, binding of this protein to the phosphonate ligand is to be expected.

Purification of alkaline phosphatases from other sources

To determine the effectiveness of the C.I. Reactive Blue 2 phosphonate derivative as a general alkaline phosphatase affinity ligand, purification from a wide variety of sources was attempted. Table V shows that the ligand is effective for both calf and bovine intestinal alkaline phosphatase even though SDS-PAGE revealed the

TABLE V

PURIFICATION OF ALKALINE PHOSPHATASES FROM A WIDE VARIETY OF SOURCES USING THE C.I. REACTIVE BLUE 2 PHOSPHONATE ANALOGUE AS THE AFFINITY LIGAND

Dialysed crude alkaline phosphatase preparation (1200 U in 4 ml 10 m*M* Tricine–NaOH buffer, pH 8.5) was applied to a small column containing 1.2 g of pre-equilibrated immobilised dye gel (2.6 μmol dye/g gel). The column was washed with 45 ml of buffer, then a 3-ml pulse of 5 m*M* KH_2PO_4 in 10 m*M* Tricine–NaOH buffer, pH 8.5 was applied. Finally any remaining protein was eluted using a 5-ml wash of 1 *M* NaCl.

Alkaline phosphatase source and type	*Specific activity of crude preparation (U/mg protein)*	*Applied alkaline phosphatase bound (%)*	*Total enzyme recovery (%)*	*Specific activity of final preparation (U/mg protein)*
Calf intestine Type I	3.0	76	103	940[a]
Bovine intestine Type I-S	8.8	44	98	850[a]
Chicken intestine Type V	8.7	6	93	350[a]
Pig intestine Type IV	1.0	18	90	107[b]
Sheep intestine Type XII	1.6	17	96	183[b]
Human placental Type XVII	8.6	1	82	40[b]
E. coli Type III-S	4.0	2	71	35[b]

[a] Bound alkaline phosphatase eluted using a 5 m*M* inorganic phosphate pulse.

[b] Bound alkaline phosphatase not eluted using inorganic phosphate pulse but would elute using 1 *M* NaCl.

contaminating proteins present in each crude preparation to be quite different. However, in all other cases both the binding ability of the alkaline phosphatase and the purification factor achieved were disappointingly low. In the case of the pig, sheep, human and *Escherichia coli* isoenzymes the alkaline phosphatase which bound was not eluted using a 5 m*M* inorganic phosphate pulse but was eluted using 1 *M* NaCl suggesting non-specific binding. The lower recoveries of the *E. coli* and human placental enzymes may be attributable to hydrophobic binding.

Affinity chromatography of alkaline phosphatase

A crude alkaline phosphatase preparation (2 g lyophilised powder dissolved in 10 ml of 10 m*M* Tricine–NaOH buffer, pH 8.5; approx. 2000 U) was applied at a flow-rate of 10 ml/h to a column (5 g moist weight gel) of agarose-immobilised C.I. Reactive Blue 2 phosphonate analogue (IV, 2.6 μmol dye/g moist weight gel). Approximately 80% of the applied activity bound to the adsorbent, with loosely bound protein being desorbed by consecutive washes with 60 m*M* Tricine–NaOH buffer pH 8.5 and 10 m*M* Tricine–NaOH buffer pH 8.5 respectively until the background absorbance at 280 nm was negligible. Elution with a linear gradient (0–5 m*M*) of inorganic phosphate (KH_2PO_4) resulted in a broad peak of alkaline phosphatase activity comprising 72% of the bound activity with an overall specific activity of 760 U/mg and representing a 250-fold purification. Subsequent application

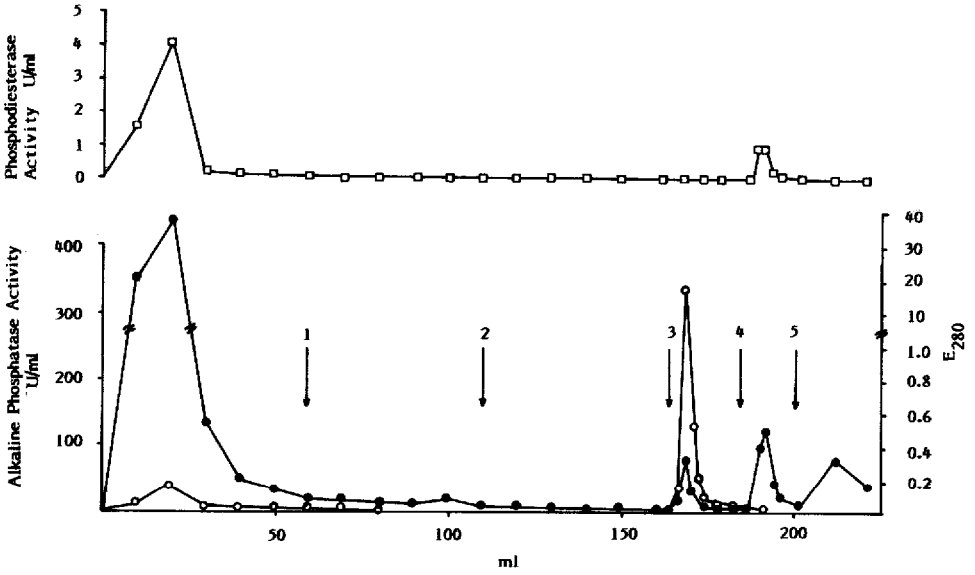


Fig. 3. Chromatography of a crude calf intestinal extract on Sepharose CL-6B-200 immobilised C.I. Reactive Blue 2 phosphonate analogue. A crude alkaline phosphatase preparation [2 g lyophilised powder/10 m*M* Tricine–NaOH buffer pH 8.5 (10 ml); approx. 2000 U] was applied at a flow-rate of 10 ml/h to a column (30 cm × 1 cm I.D.; 5 g moist weight gel) of Sepharose CL-6B-200 immobilised phosphonate analogue (2.6 μmol/g moist weight gel) equilibrated with 10 m*M* Tricine–NaOH buffer pH 8.5 at 4°C. The column was washed at the points indicated as follows: (1) 60 m*M* Tricine–NaOH buffer pH 8.5; (2) 10 m*M* Tricine–NaOH buffer pH 8.5; (3) 5 m*M* KH_2PO_4 (20 ml); (4) 20 m*M* KH_2PO_4 (15 ml); (5) 0.75 *M* KCl. Fractions (2 ml) were analysed for alkaline phosphatase activity (○), phosphodiesterase activity (□) and protein (E_{280}, ●).

of a pulse (15 ml) of inorganic phosphate (20 m*M*) released the adsorbed phosphodiesterase. Finally, the column was flushed with 0.75 *M* KCl to desorb all remaining protein and then re-equilibrated with 10 m*M* Tricine–NaOH buffer pH 8.5 prior to reuse.

Fig. 3 shows that repetition of the chromatographic run with a pulse (20 ml) of inorganic phosphate (5 m*M*) in place of the gradient elution, resulted in the appearance of alkaline phosphatase activity in a sharp peak containing 89% of the bound enzyme with an overall specific activity of 870 U/mg (290-fold purification). Over 60% of the eluted activity was contained in a single fraction (2 ml) with a specific activity in excess of 1000 U/mg and representing a 330-fold purification of the enzyme from the crude preparation in a single step. SDS-PAGE (Fig. 4) and determination of the specific activity revealed that alkaline phosphatase purified using affinity chromatography on the immobilised phosphonate analogue of C.I. Reactive Blue 2 was of equivalent purity to commercially available "high-purity" preparations which had specific activities of 850–1250 U/mg under the assay conditions used in this work. In addition, Fig. 3 shows that specific elution using inorganic phosphate (5 m*M*) separated the alkaline phosphatase activity from the phosphodiesterase activity, which was eluted using higher inorganic phosphate concentrations (20 m*M*). The final alkaline phosphatase preparation contained <0.05 U phosphodiesterase/mg protein. The

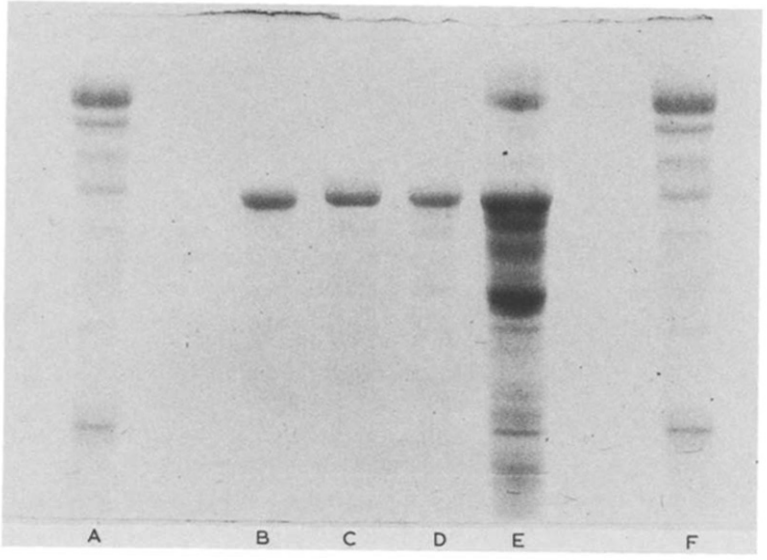


Fig. 4. Comparison of the C.I. Reactive Blue 2 phosphonate purified enzyme with commercial "high purity" preparations by SDS-PAGE. For the comparison equivalent amounts of dye-purified and commercial enzyme were applied to the gel. Tracks A and F, bovine phosphodiesterase I standard; tracks B and C, commercial "high-purity" alkaline phosphatase preparations; track D alkaline phosphatase purified by affinity chromatography on the immobilised phosphonate adsorbent with a specific eluent of inorganic phosphate (5 m*M*; Fig. 3); track E, profile of the total protein binding to the immobilised phosphonate adsorbent from the crude calf intestinal alkaline phosphatase preparation obtained by elution with 0.75 *M* KCl. Electrophoresis of crude calf intestine alkaline phosphatase preparation is not shown since it results in a single, heavily stained band corresponding to bovine serum albumin which accounts for *ca.* 30% of the total protein in the crude extract.

adsorbent could be re-used many times with no apparent reduction in capacity or purification performance being observed over a 12-month period of regular usage. It should also be noted that the alkaline phosphatase loadings used in this process (1000 U/5 ml) are far higher than those reported in other studies. This factor, together with the stability and ease of synthesis and immobilisation makes this process particularly suitable for large scale commercial application.

DISCUSSION

Immobilised triazine dyes have been successfully exploited for the purification of a host of complementary proteins[5], although only in a few cases is the dye–protein interaction well understood[21,22]. The present study indicates that the affinity of calf intestinal alkaline phosphatase for terminal ring analogues of C.I. Reactive Blue 2 (Fig. 1) is governed largely by the anionic substituent on the terminal ring. Substitution of anionic functions such as carboxylate and phosphonate for the ubiquitous sulphonate in commercially available dyes, dramatically enhances the specificity of the dye–protein interaction, presumably because both are competitive inhibitors and bind to the active site of the enzyme[3,16]. It is probable that under the binding conditions used in this study the ligand interacts directly at the active site since the enzyme is specifically eluted with low concentrations of substrates and inhibitors. However, the lack of detailed knowledge of the calf intestinal alkaline phosphatase active site, such as X-ray crystallography data, makes absolute conclusions regarding the dye–protein interaction very difficult. In addition, further rational design of the ligand in order to maximise presentation of the phosphonate or to determine the contribution of the other components of the dye moieties to the dye-protein interaction will be dependent on the availability of such data.

Binding of alkaline phosphatase to the immobilised C.I. Reactive Blue 2 phosphonate ligand appears to be highly ionic in nature since the interaction is greatly affected by pH and ionic strength, the enzyme is easily desorbed using low salt concentrations and is not eluted using 50% (v/v) glycerol. In addition, the uncharged aniline derivative shows very low affinity for the enzyme. Kirchberger *et al.*[23] also found that the binding of calf intestinal alkaline phosphatase to Procion Red HE-3B was mainly stabilised by electrostatic and not hydrophobic interactions.

Phosphonate[3] and arsonate[1] derivatives have been used previously for the purification of alkaline phosphatase. In both cases, presentation of the anionic competitive inhibitors attached to a bulky aromatic/hydrophobic moiety proved the most effective ligands. Similar conclusions have been drawn for the purification of acid phosphatase[24]. Phosphonate derivatives proved superior to arsonate presumably because benzyl phosphonate is iso-spatial to the very high affinity substrate, phenyl phosphate[17].

The use of the Sepharose-immobilised phosphonate analogue of C.I. Reactive Blue 2 in conjunction with specific elution with inorganic phosphate resulted in a calf intestinal alkaline phosphatase preparation purified 330-fold in a single step from a crude extract. Absolute comparisons with results reported by other workers are difficult since quoted specific activities depend on the enzyme assay used and the method of protein determination. For example, use of a phenyl arsonate adsorbent and specific elution with inorganic phosphate probably achieved a homogeneous

preparation of calf intestinal alkaline phosphatase[1]. However, the starting material was a partially purified extract and phenyl arsonates are more labile than phosphonates[2]. Other workers have exploited an immobilised phosphonate derivative to effect an 11-fold purification of calf intestinal alkaline phosphatase[3] and a 400-fold purification of human liver alkaline phosphatase[4] respectively. Commercial dye adsorbents have been exploited by a number of workers for alkaline phosphatase purification. Bouriotis and Dean[6] obtained a 295-fold purification of calf intestinal alkaline phosphatase using α-naphthyl phosphate as specific eluent. In this case, hydrolysis of the organic phosphate eluent led to contamination of the resulting enzyme preparation with α-naphthol which was removed by dialysis. Also, the use of sequential affinity chromatography on immobilised Cibacron Blue F3G-A and Procion Red HE-3B as components in a multi-step purification procedure resulted in apparent homogeneity of calf intestinal alkaline phosphatase[7] and a single step 380-fold purification of human intestinal alkaline phosphatase was achieved using affinity chromatography on immobilised Remazol Yellow GGL[8].

Although these affinity purification techniques are extremely effective in some cases, they cannot be considered generally applicable to alkaline phosphatase purification since this and other studies has shown that alkaline phosphatase isoenzymes differ markedly in their affinity for such ligands[25]. For example, the human placental enzyme shows very low affinity for the C.I. Reactive Blue 2 phosphonate ligand and other immobilised triazine dyes. However, Yasmin and Qadri[10] did obtain a 46-fold purification by precipitating the enzyme onto a column of Cibacron Blue 3G-A-Sepharose 4B using a 50% (w/v) saturated ammonium sulphate solution. Elution was then achieved by lowering the salt concentration. Similarly, Smith and Peters[9] achieved a 244-fold purification of the human polymorphonuclear leukocyte enzyme using solubilisation with Triton X-100 and chromatography on immobilised Cibacron Red F. The bound enzyme was not eluted using high salt concentrations or specific eluents. In these two examples the exact nature of the dye–protein interaction is unknown but is likely to involve mainly hydrophobic rather than ionic interactions. Hydrophobic binding by the human placental and the *E. coli* alkaline phosphatases may account for their low recoveries when screened for their ability to bind the Reactive Blue 2 phosphonate ligand. The inability of isoenzymes, such as the human placental, to bind to triazine dyes under low salt conditions may be due to their high sialic acid content which would increase their overall negative charge and possibly prevent binding to anionic ligands[26].

CONCLUSIONS

The phosphonate terminal ring analogue of C.I. Reactive Blue 2 represents only the second example of the use of specifically designed biomimetic dyes for protein purification[11]. Frontal analysis experiments showed the phosphonate dye adsorbent to be more selective for alkaline phosphatase binding and have a higher binding capacity than the most effective of the commercial dyes tested. Its use in conjunction with specific elution with inorganic phosphate resulted in a single step 330-fold purification of the calf intestinal enzyme from a crude preparation. The dye purified enzyme was of equivalent purity to commercial high purity preparations by SDS-PAGE and specific activity comparisons and contained <0.05 U phospho-

diesterase/mg protein. It is believed that this is the first study in which affinity purified calf intestinal enzyme has been compared directly with commercial "high-purity" preparations.

The phosphonate ligand retains the advantages of commercial dyes in terms of cost, stability, reusability, sterilizability and ease of immobilisation whilst displaying greatly improved specificity for the target protein. It is anticipated that as more information concerning the detailed architecture of enzyme active sites and the nature of enzyme ligand interactions becomes available, the flexibility of dye design and synthesis, particularly using computer aided molecular graphics will accelerate the development of this new approach to affinity chromatography.

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