Monosized adsorbents for high-performance affinity chromatography

Application to the purification of calf intestinal alkaline phosphatase and human urine urokinase

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(First received August 9th, 1990; revised manuscript received November 6th, 1990)

ABSTRACT

    Affinity adsorbents comprising monodisperse spherical synthetic macroporous beads offer the prospect of high-capacity, high-resolution separation of proteins at low operating pressures. Purpose-designed biomimetic dyes were covalently attached to Dynospheres XP-3507 beads and exploited for the purification of calf intestine alkaline phosphatase and human urine urokinase from crude extracts. This study demonstrates that the combination of specifically designed affinity ligands with monosized support materials is a powerful approach to the resolution of proteins by high-performance affinity chromatography.

INTRODUCTION

    Affinity chromatography is now firmly established in most modern enzyme purification protocols [1–4]. However, increasingly stringent demands on the purity of protein preparations, particularly for therapeutic applications, have resulted in numerous refinements to the original technique. For example, the exquisite specificity of affinity chromatography has now been combined with the speed and resolving power of high-performance liquid chromatography to create a technique known as high-performance affinity chromatography [5,6]. This approach is finding increasing application in enzyme purification, especially in cases where the affinity ligand employed is a triazine dye [7–11]. Adsorbents based on commercially available dye-ligands offer many well documented advantages over those based on truly biospecific ligands but can suffer from lower specificity [12,13]. However, more recently, it has been shown that purpose-designed dye-ligands can offer higher specificity for complementary enzyme molecules, marking a new era in the downstream processing of

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proteins [14–16]. For example, specifically designed analogues of C.I. Reactive Blue 2 were effective in the purification of horse liver alcohol dehydrogenase [14] and a terminal ring-phosphonate analogue of C.I. Reactive Blue 2, when immobilised to Sepharose CL-6B, was shown to achieve a spectacular single-step 330-fold purification of calf intestine alkaline phosphatase [16]. Similarly, a novel immobilised cationic triazine dye was highly selective for binding trypsin-like proteases and could completely resolve trypsin from chymotrypsin in a crude pancreatic extract [15]. In the present study, the effectiveness of monosized affinity adsorbents to purify alkaline phosphatase and urokinase from crude preparations of calf intestine and human urine respectively has been investigated. Previous studies have shown that affinity adsorbents based on monodisperse beaded support materials such as Dynospheres XP-3507 exhibited high capacity for complementary proteins and could operate at very low back pressures [10]. In the present study, purpose-designed dye ligands (Fig. 1, I and IV) were immobilised to similar monosized synthetic macroporous spherical particles, Dynospheres XP-3507. For comparative purposes two other ligands (Fig. 1, II and III), both known to interact with alkaline phosphatase and urokinase respectively, were also immobilized on the same monosized matrix and tested.

EXPERIMENTAL

Materials

Alkaline phosphatase (calf intestine, typically 3 units mg^{-1} protein), urokinase (human urine, 0.1 Sigma units mg^{-1} protein), glycine, p-nitrophenylphosphate, tricine, 4-aminobenzylphosphonic acid (Fig. 1, **III**), 1,6-diaminohexane, 1,1'-carbonyldiimidazole, glycerol, 3-(N-morpholino)propanesulphonic acid (MOPS) and Trizma base (Tris) were purchased from Sigma (U.K.). The urokinase substrate S-2444 was from KabiVitrum, Sweden. The terminal ring-phosphonate analogue of C.I. Reactive Blue 2, C.I. Reactive Blue 2 and the cationic dichlorotriazine dye were gifts
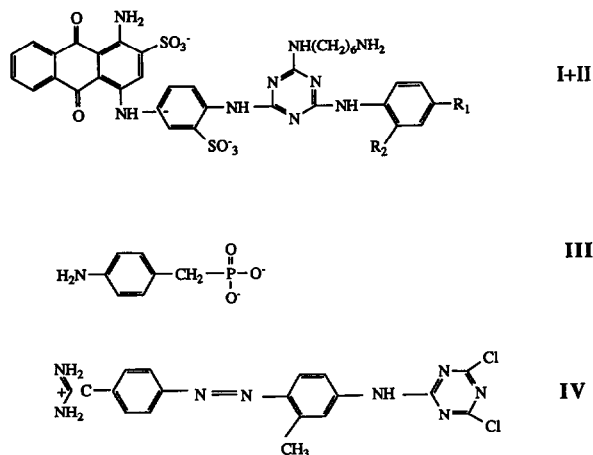

Fig. 1. The chemical structures of the affinity ligands used in this study. **I**: $R_1 = -CH_2PO_3^-$ and $R_2 = -H$, 6-aminohexyl terminal ring-phosphonate C.I. Reactive Blue 2; **II**: $R_1 = -H$ and $R_2 = -SO_3^-$, 6-aminohexyl C.I. Reactive Blue 2; **III**: 4-aminobenzylphosphonic acid; **IV**: cationic dichlorotriazine dye.

from Dr. C. V. Stead (Dyestuffs Consultancy Service, Manchester, U.K.) and were synthesized according to a published procedure [17]. The monosized support materi-al, Dynospheres XP-3507 (20 μm, 50–2000 Å, 100 m$^2 \cdot$ g^{-1}) was a much appreciated gift from Dyno Particles (Norway). The hydrophilic membrane filters (Durapore, Millex-GV, 0.22 μm) were from Millipore (U.K.).

Equipment
The automated chromatography system employed was from Pharmacia (U.K.) and consisted of two P-500 pumps, a chromatographic controller (LCC-500), one motor valve with load/injection/washing facility (MV-7), two motor valves for multi-column selection (MV-8), a UV-M absorbance monitor (280 nm), a dual-channel recorder and a fraction collector (FRAC-100). Enzyme assays were performed in a Perkin-Elmer lambda 5 dual-beam UV–VIS spectrophotometer with a Peltier tem-perature controller at the sample position.

Assays
Alkaline phosphatase activity was monitored at 405 nm following the hydroly-sis of the substrate *p*-nitrophenylphosphate, resulting in an absorbance increase due to the production of *p*-nitrophenolate. A total assay volume of 1 ml contained: gly-cine–NaOH buffer, 0.1 mmol, pH 10.4; *p*-nitrophenylphosphate, 6 μmol; magnesium chloride, 1 μmol; zinc chloride, 1 μmol; alkaline phosphatase, typically 0.15 units. One unit of enzyme activity is defined as the amount of enzyme which catalyses the formation of 1 μmol *p*-nitrophenolate anion per minute at 37°C.

Urokinase activity was monitored at 405 nm following the hydrolysis of the tripeptide S-2444, resulting in an absorbance increase due to the production of *p*-nitroaniline. A total assay volume of 1 ml contained: Tris–HCl buffer, 0.05 mmol, pH 8.8; NaCl, 0.038 mmol; S-2444, 0.15 mg; urokinase, typically 0.05 units, 0.2 mg protein. One unit of enzyme activity is defined as the amount of enzyme which cata-lyses the formation of *p*-nitroaniline causing a change of absorbance at 405 nm of 1 per minute at 37°C.

The protein content was determined by measuring the absorbance at 280 nm using a mass extinction coefficient of 1.0 absorbance unit per mg ml^{-1} for alkaline phosphatase [16], and 1.15 absorbance units per mg ml^{-1} for urokinase.

Preparation of monosized affinity gels
Synthesis of affinity ligands I and II. To a 50-ml flask were added 1,6-diamino-hexane (1.45 g, 12.5 mmol), water (20 ml) and a solution of phosphonated C.I. Reactive Blue 2 [16] for preparing I or C.I. Reactive Blue 2 for preparing II (15 ml, 0.5 g dye, approx. 0.5 mmol). The reaction mixture was incubated at 60°C under shaking for 2–3 h and then slowly poured into 0.2 *M* HCl (300 ml) under gentle stirring, and left overnight for complete precipitation of the 6-aminohexyl dye. The product was washed on a filter paper (Whatman, hardened 52) with 0.2 *M* HCl (150 ml) and acetone (150 ml), and dried in a drying chamber (approx. 40°C) for 30 min.
Immobilisation of the affinity ligands I, II and III to Dynospheres XP-3507 parti-cles. Monosized Dynospheres particles XP-3507 (20 μm, 50–2000 Å, 100 m^2 g^{-1}) carry free hydroxyl groups which were activated with 1,1'-carbonyldiimidazole prior to coupling to ligands containing free amino groups. Particles (3 g) were washed on a

sintered glass funnel (porosity 4) with water, acetone and dry acetone or acetonitrile, transferred to a 50-ml conical flask containing dry acetonitrile (25 ml) and 1,1'-carbonyldiimidazole (1.5 g) added immediately. The suspension was left to react under shaking for 30 min whence the activated particles were washed with acetone and sucked-dry on a glass funnel.

Immobilisation of the dye-ligands to the activated particles was performed as follows: sucked-dry activated particles (1.5 g) were added to a solution of ligand (150 mg) in sodium bicarbonate (0.1 M, 8 ml). The suspension was sonicated (1–2 min) under reduced pressure, and left to react at 20–25°C under shaking for 4 days. The particles were finally washed on a sintered glass funnel (porosity 4) with water, 2 M NaCl, and water until no free ligand was evident in the washings (5.6 μmol of **I** and 9.1 μmol of **II** immobilised g^{-1} wet weight particles).

Immobilisation of the cationic dichlorotriazine dye to Dynospheres XP-3507 particles. Particles (1.4 g) were washed on a sintered glass funnel (porosity 4) with water, water–dimethylformamide (DMF) (1:1, v/v), DMF, DMF–triethylamine (99.04:0.96, v/v), sucked dry, and transferred into a 50-ml flask containing triethylamine (0.135 ml, 0.96 mmol) and cationic dye **IV** (17 mg) in DMF (14 ml). The suspension was sonicated for 30 s and incubated at 40°C under shaking for 16 h. The dyed particles were washed on a sintered glass funnel (porosity 4) with DMF until no dye was evident in the washings, followed with DMF–water (1:1, v/v) and water (11.4 μmol cationic dye g^{-1} wet weight particles).

All determinations of immobilised ligand concentration were performed by the gel-suspension method [10], using solvent water–glycerol (1:1, v/v) for the anionic dyes and DMF–glycerol (1:1, v/v) for the cationic dye. The molar extinction coefficient was taken as 12 500 l mol^{-1} cm^{-1} for dyes **I** and **II**, and 13 900 mol^{-1} cm^{-1} for the cationic dye **IV**.

Chromatographic procedures

All chromatographic procedures were performed at room temperature using pressure-resistant glass columns (100 bar, 5.0 × 0.5 cm, HR 5/5 type, Pharmacia, Sweden) packed with the affinity adsorbents to 1 ml packed bed volume.

A commercial crude calf intestine alkaline phosphatase powder (1.6 g, 3 units mg^{-1} according to the supplier) was dissolved in tricine–NaOH buffer, 10 mM, pH 8.0 (8 ml) and dialysed against 2 l of the same buffer for at least 14 h at 4°C. The dialysate was filtered through a membrane filter (Durapore, Millex-GV, 0.22 μm) and contained, typically, alkaline phosphatase activity of 288 U mg^{-1} and total protein 110 mg ml^{-1}. The sample (approx. 9 ml) was continuously applied at a flow-rate of 0.1 ml min^{-1} (0.5 cm min^{-1}) to the affinity column (ligands **I**, **II** and **IV**) which had previously been equilibrated with the above buffer. After sample application, the flow-rate was increased to 0.5 ml min^{-1} (2.5 cm min^{-1}) and the column was washed with irrigating buffer until the absorbance (280 nm) of the effluents was less than 0.02. Adsorbed alkaline phosphatase was specifically eluted with inorganic phosphate (potassium dihydrogenorthophosphate, 6 ml, 6 mM in tricine–NaOH buffer, 10 mM, pH 8.0). Remaining activity and other bound proteins could subsequently be eluted with NaCl (approx. 10 ml, 1.5 M). Fractions (2 ml) collected during the specific elution step and containing enzyme activity were pooled and both the total enzyme activity (units) and protein content (mg) were determined.

Commercial crude human urine urokinase was dissolved in MOPS–NaOH buffer, 20 mM, pH 7.0 (2 mg solids or protein ml^{-1}, 0.26 units mg^{-1}) and filtered through a membrane filter (Durapore, Millex-GV, 0.22 μm). A samples (0.2 ml, 0.104 units, 0.4 mg protein) was applied to the cationic dye adsorbent equilibrated in the same buffer at a flow-rate of 1 ml min^{-1}. The columns was washed with buffer (4 ml) to remove unbound material followed by 1 M NaCl (4 ml) to desorb non-specifically bound proteins. Adsorbed urokinase was eluted with 2 M NaSCN (6 ml). All fractions (2 ml) were tested for enzyme activity and protein content, and those with urokinase activity were pooled and the total urokinase activity and protein were determined.

RESULTS AND DISCUSSION

The technique of high-performance affinity chromatography currently enjoys popularity in protein and enzyme purification [6–8,11]. To date, however, microparticulate silica has been the matrix of choice but suffers from poor stability and requires functionalisation prior to ligand coupling [5,6,8,11]. Consequently, attention is now being directed towards rigid synthetic hydrophilic macroporous packings which
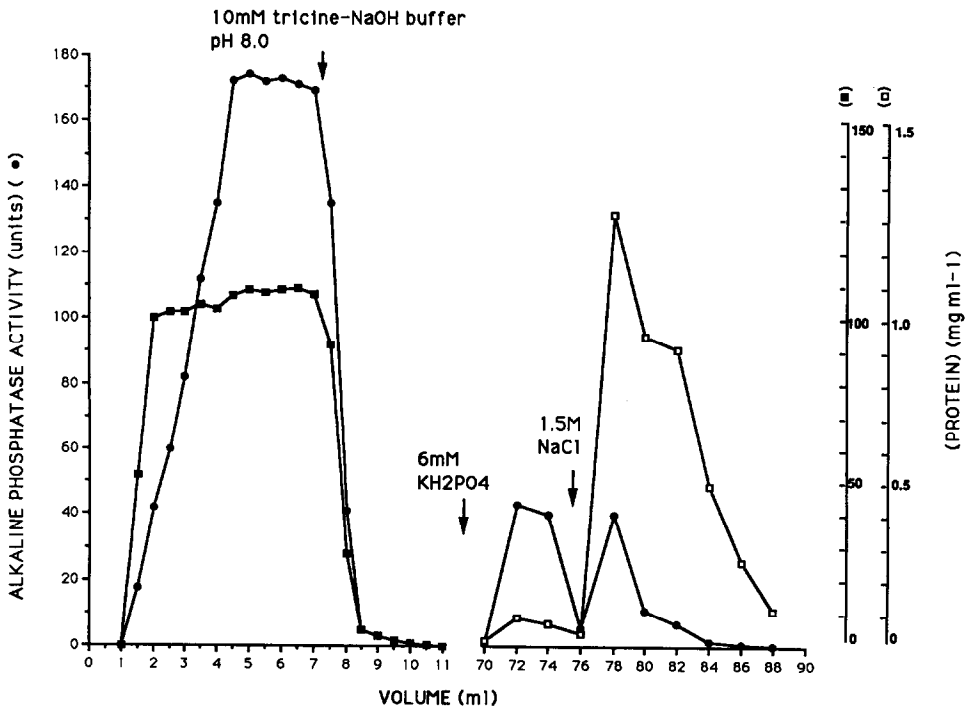

Fig. 2. A typical elution profile obtained from a chromatographic run to determine the capacity, the purification and the yield achieved for alkaline phosphatase with the affinity adsorbent Dynospheres XP-3507–immobilised 6-aminohexyl terminal ring-phosphonate C.I. Reactive Blue 2 (Fig. 1, I, 5.6 μmol dye per g particles, 5.0 × 0.5 cm bed). The material applied was a crude alkaline phosphatase extract from calf intestine (9 ml, typically 2.6 units per mg protein). For details see *Chromatographic procedures* section.

TABLE I

PERFORMANCE SUMMARY OF THE HIGH-PERFORMANCE ADSORBENTS FOR ALKALINE PHOSPHATASE FROM A CALF INTESTINAL EXTRACT CONTAINING 3 UNITS mg^{-1} PROTEIN, AND UROKINASE FROM A CRUDE HUMAN URINE PREPARATION CONTAINING 0.26 UNITS mg^{-1} PROTEIN

Enzyme	Adsorbent (ligand immobilised)	Total capacity (mg protein ml^{-1} adsorbent)	Specific capacity (units ml^{-1} adsorbents)	Purification (fold)	Specific activity (units mg^{-1})	Recovery (%)
Alkaline phosphatase	I	8.1	150	115 (140[a])	300 (420[a])	58 (41[a])
	II	9.1	10	1.3	4	50
	III	1.5	12	18	51	71
Urokinase	IV	–	–	13	3.3	290

[a] Peak activity fraction.

appear to be promising for larger scale applications [2,10]. Monosized (monodis-perse) synthetic packings such as Dynospheres XP-3507 are copolymers of ethyl-eneglycol dimethacrylate and hydroxylethyl methacrylate. Various ligands can, there-fore, be immobilised through the functional hydroxyls of this support. In earlier work, the highly reactive dichlorotriazinyl dye, Procion Blue MX-R, was immobilised directly to Dynospheres XP-3507 and the resultant affinity adsorbent used successful-ly to purify L-lactate dehydrogenase from a crude rabbit muscle extract [10]. Dy-nospheres XP-3507 display exceptionally low back-pressure (approx. 2 bar at 1 ml min^{-1} in water, 20 μm particles) due to their monodispersity and are both hydrophil-ic and macroporous (50–2000 Å). Such features are ideal for large-scale use since a high flow-rate at low back-pressure encourages high throughput.

Fig. 1 shows the chemical structures of the ligands used in this study. Mono-chlorotriazine dyes give low substitution levels when immobilised directly to silica or synthetic HPLC supports [8]; thus, an aminohexyl spacer molecule has been attached to the ligands in order to improve their immobilisation level. The new ligands, I and II, were efficiently immobilised in mild alkaline conditions through their free terminal aliphatic amino-group to Dynospheres XP-3507 which had previously been activated with 1,1'-carbonyldiimidazole, whilst ligand III was immobilised less efficiently, through its aromatic amino-group. The highly reactive cationic dichlorotriazine dye IV displays low water solubility and was immobilised to the particles in an organic solvent system.

The ability of the adsorbents to function as high-performance media was eval-uated with commercial crude preparations of two selected enzymes, calf intestinal alkaline phosphatase and human urine urokinase. The ligands I and cationic dye IV were purpose-designed for the two enzymes used here. Fig. 2 shows a typical elution profile, whereas Table I summarises the capacity, purification and recovery of alka-line phosphatase from the different monosized affinity adsorbents. Two capacity fig-ures were used; the total capacity is expressed as mg of total protein per ml adsorbent, whilst the specific capacity is expressed as units of total alkaline phosphatase per ml adsorbent. In both cases, the protein was eluted with inorganic phosphate followed by NaCl from a saturated 1-ml adsorbent. The purification is expressed as fold of increase in specific activity over the starting material (2.6 mg ml^{-1}) of the pooled fractions containing enzyme activity and obtained by specific elution with inorganic phosphate. The recovery is expressed as percentage over the total enzyme activity desorbed in specific and salt elution. The purpose-designed affinity ligand I achieved a substantially higher purification (115-fold) than the other two ligands, II and III, which gave purifications of only 1.3- and 18-fold, respectively. The peak-activity fraction from the adsorbent with the ligand I contained approximately 70% of the specifically eluted activity and achieved a greater than 140-fold purification. Increas-ing the concentration of specific eluent, inorganic phosphate, did not effect an appre-ciable increase in enzyme recovery from the adsorbent. Concentration of phosphate above 10 mM resulted in alkaline phosphatase of reduced purity due to co-elution of other bound proteins [16]. Following elution with phosphate, an appreciable propor-tion (42%) of bound enzyme was eluted with 1.5 M NaCl. It is likely that non-specific adsorption of the enzyme to the charged adsorbents may account for these observa-tions, since it is known that the binding of alkaline phosphatase to the immobilised C.I. Reactive Blue 2 phosphonate analogue is highly ionic in character [16]. The

slightly higher capacity observed with adsorbent **II** can be attributed to its higher immobilised ligand concentration. Direct comparison of the performance of the previously reported agarose-immobilised terminal ring phosphonate analogue of C.I. Reactive Blue 2 [16] and Dynospheres-immobilised ligand **I** is difficult because the former work was performed with an affinity adsorbent bearing no spacer molecule and at 4°C and pH 8.5. In contrast, the present work introduced a hexamethylene spacer into the affinity ligand **I** in order to improve the coupling yield, and was executed at room temperature and pH 8.0. The reasons for doing so were two-fold: room temperature is economical and convenient for a production process, and at pH 8.5 only trace amounts of enzyme were adsorbed to the immobilised **I** affinity column. The previously reported agarose adsorbent [16] achieved up to a 330-fold purification, although the procedure required at least twice the time as the present high-performance affinity packing. To this end, high purification is often sacrificed in order to achieve high throuput in production processes.

Urokinase, a trypsin-like protease, is an expensive and important thrombolytic agent which binds tightly to the potent inhibitor benzamidine [18]. Fig. 3 shows that it is possible to purify crude urokinase by 13-fold in a single step simply by passing a crude sample down the benzamidine-bearing cationic dye high-performance column.
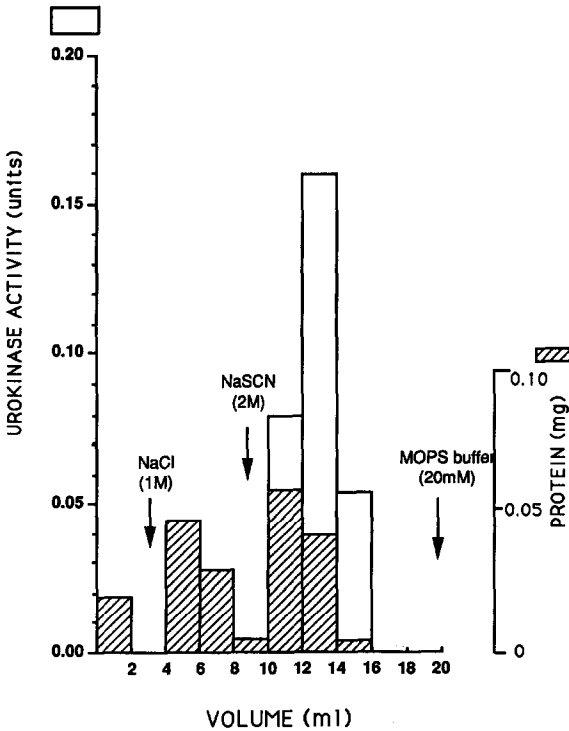

Fig. 3. The elution profile of a chromatographic run to purify urokinase on the affinity adsorbent Dynospheres-immobilised cationic dichlorotriazine dye (Fig. 1, **IV**, 11.4 μmol dye per g particles, 5.0 × 0.5 cm bed). The material processed was crude urokinase from human urine (0.2 ml, 0.104 units, 0.4 mg protein). For details see *Chromatographic procedures* section.

After removing non-specifically bound proteins with 1 M NaCl, the tightly bound urokinase was eluted with 2 M NaSCN in a yield of 290% based on activity measurements (Table I). The high recovery is consistently observed and is probably due to the removal of inhibitors present in the crude starting preparation.

This study demonstrates the value in combining specifically designed affinity ligands with monodisperse support materials in order to generate powerful new adsorbents for the resolution of proteins by high-performance affinity chromatography.

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