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Use of metal chelate affinity chromatography for removal of zinc ions from alkaline phosphatase from *Escherichia coli*

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ABSTRACT

Alkaline phosphatase from *Escherichia coli* (APEC) is not retained at 4°C on a metal-free tris(carboxymethyl)ethylenediamine (TED) column, but at 15°C the metalloenzyme becomes bound to the gel. Chromatography of phosphatase on metal-free TED gel indicates a decline in its enzymic activity and zinc content to about 26% and 40%, respectively. The activity of chromatographed APEC can be partially restored by addition of zinc ions, indicating that metal-free TED gel is capable of removing zinc ions from alkaline phosphatase.

INTRODUCTION

Immobilized metal affinity chromatography (IMAC) is commonly used not only for protein purification, but also for the topography of reactive residues on the surface of proteins (for reviews see refs. 1–3). In "regular" IMAC the metal, mostly chelated by iminodiacetate (IDA) to the gel, interacts with amino acid residues of the protein. In "reversed" IMAC the metal, a structural component of the protein, interacts with an immobilized chelator. This type of chromatography has not been exploited very extensively. In "reversed" IMA adsorption a long spacer arm is probably essential for the chelating group to reach the metal located internally in the metalloprotein [4].

Porath and Olin [5] described the synthesis of tris(carboxymethyl)ethylenediamine (TED)–agarose. Compared with IDA–agarose, the TED gel is characterized by longer spacer arms and makes stabler complexes with metal ions. Passage of carboxypeptidase A (Zn^{2+}–CPD) through metal-free TED gel abolished the peptidase activity of the enzyme. The activity can be fully restored by adding zinc ions [6]. The results strongly suggested that TED–agarose exhibits a strong affinity to metal ions, and therefore could be used for removing the metal from some proteins. In this work, to confirm this suggestion, attempts to remove zinc ions from another metalloprotein, alkaline phosphatase from *Escherichia coli* (APEC), were made. The chromatography of APEC on metal-free TED gel was followed by determination of its zinc content and enzymatic activity.

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EXPERIMENTAL

Materials

MES, Trizma Base (analytical-reagent grade), *p*-nitrophenyl phosphate (pNPP), alkaline phosphatase from *E. coli* (APEC) and EDTA were obtained from Sigma. Tris(carboxymethyl)ethylenediamine–Sepharose 6B (TED gel) was a kind gift from Professor J. Porath (Uppsala University). PD-10 columns were obtained from Pharmacia and the protein assay dye solution from Bio-Rad Labs. All other reagents were of analytical-reagent grade.

Methods

Preparation of columns. The chromatographic adsorbent (TED–Sepharose 6B) was packed into the columns (3.3 × 1.0 cm I.D., $V_t = 2.8$ ml) in distilled water and equilibrated with 50 m*M* MES (pH 5.5), then 1 mg of the enzyme in 1.2 ml of equilibration buffer was applied to the column. The column was washed with 0.5 ml of the same buffer and allowed to stand for 1 h. Washing with the same buffer was then continued until all of the non-adsorbed material was eluted from the column; at a flow-rate 6 ml/h, fractions of 0.5 or 1 ml were collected. In the case of protein binding on the gel, sodium chloride was included for the elution.

Dialysis of alkaline phosphatase (APEC) against EDTA. Treatment of EDTA (by dialysis) was performed under the described conditions [7], with slight modification. Holoenzyme dissolved in 50 m*M* MES (pH 6.0) up to a protein concentration of 0.5 mg/ml was dialysed at 25°C against a 400-fold volume excess of 10 m*M* Tris–HCl (pH 8) containing 10 m*M* EDTA. After dialysis the protein was filtered on PD-10 columns previously equilibrated with 10 m*M* Tris–HCl (pH 8).

Removing of metal contamination. Adventitious metal ions, especially zinc, present in trace amounts in water, buffers and eluents were eliminated by passing all these solutions through metal-free TED gel (22 × 1.3 cm I.D. column), at a flow-rate of 18 ml/h at room temperature. To metal-free TED gel, 3 m*M* substrate solution (pNPP) was added. The suspension was kept in the dark for 16 h at 4°C, with occasional shaking, then the gel was removed by centrifugation. For storage of protein fractions and determination of enzymatic activity all tubes, pipette tips, cuvettes and vessels were made of plastic and before use were thoroughly washed with 10 m*M* EDTA, water, 10 m*M* sulphuric acid and finally metal-free water.

Determination of activity of alkaline phosphatase from E. coli (APEC). To 0.9 ml of 1 *M* Tris–HCl buffer (pH 8), 0.1 ml of enzyme solution (containing 1–6 μg of protein) and 0.5 ml of substrate were added. Each sample was incubated for 10 min at 37°C, cooled in an ice-bath and the amount of product formed was measured directly at 410 nm. To parallel samples a 5-molar excess of zinc sulphate (calculated on the phosphatase concentration) was added. Phosphatase activity is expressed in units (U), where 1 U is that activity liberating 1 μmol of *p*-nitrophenol per minute in 0.6 *M* Tris–HCl (pH 8)–1 m*M* pNPP at 37°C.

Protein determination. Protein was determined according to Bradford [8] with bovine serum albumin as a standard.

Zinc determination. Zinc was determined, after wet combustion, in a Perkin-Elmer 300 atomic absorption spectrometer. The zinc content was calculated based on the molecular weight of a subunit (43 000).

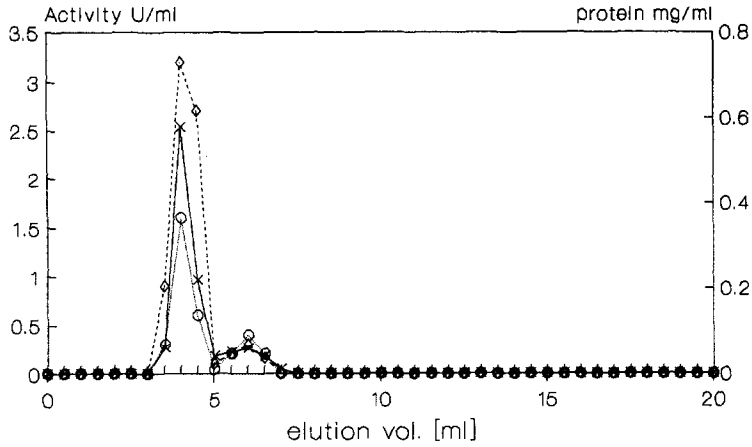


Fig. 1. Chromatography of alkaline phosphatase on metal-free TED gel at 4°C. The column was washed with 0.05 *M* MES (pH 5.5). X = Protein; enzyme activity (○) in the absence and (◇) in the presence of zinc ions.

RESULTS

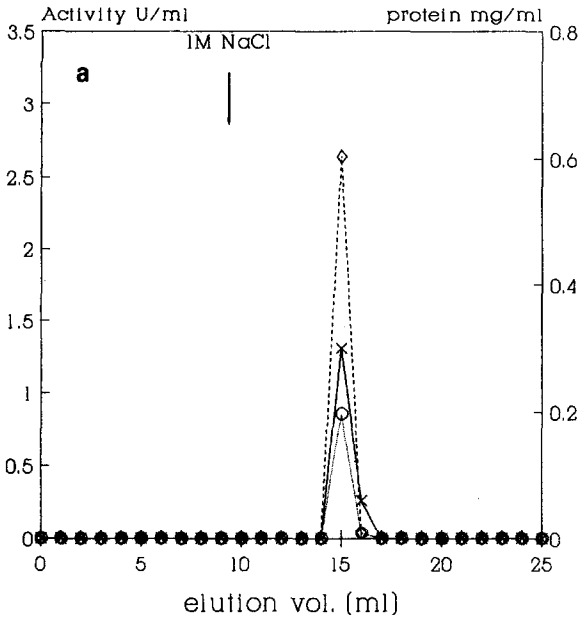
Commercial APEC did not bind to metal-free TED gel at 4°C and enzymatic protein was eluted in the equilibration buffer [0.05 *M* MES (pH 5.5)] (Fig. 1). However, when the chromatography was performed at elevated temperature (15°C) the enzyme was retained on the column and its elution was possible by inclusion of sodium chloride (Fig. 2a). Using a linear gradient of the salt APEC emerged from the chelated column at a 0.2 *M* concentration of sodium chloride (Fig. 2b).

Passage of APEC through the metal-free TED gel reduced its enzymatic activity to about 26% or 10% of the initial value in chromatography at 4 and 15°C, respectively. Also, the zinc content of the enzyme decreased markedly, from *ca.* 3 mol per enzyme subunit initially to 1.1–1.2 mol after chromatography on the metal-free gel (Table I).

The chromatographed enzyme was partially reactivated, to 50–60% of its initial level, by addition of Zn^{2+} to the incubation sample. "Short" dialysis (90 min) against soluble chelating agent did not decrease the zinc content of the chromatographed enzyme (data not shown), whereas "prolonged" dialysis (12 h) of native enzyme reduced its zinc content to about 0.5 mol per monomer of enzyme (Table I).

DISCUSSION

APEC is a dimeric metalloenzyme with two reactive centres. The reactive centre per monomer of the enzyme contains three metal sites; two metal sites are occupied by zinc and the third by magnesium or, in the absence of magnesium, by zinc [9]. These results are consistent with our data. As can be seen from the present results, the native form of APEC contains about three metal ion per monomer of the enzyme. Passage of the enzyme through a metal-free TED column reduces the amount of the metal to about one atom with a simultaneous decrease in phosphatase activity. Earlier studies



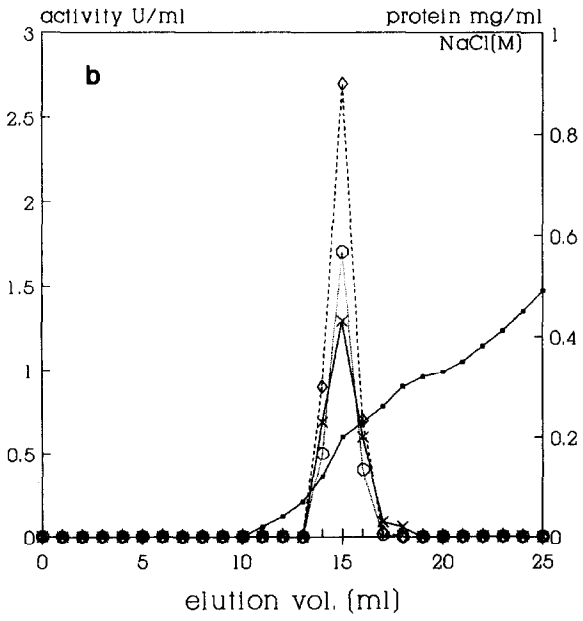


Fig. 2. Chromatography of alkaline phosphatase on metal-free TED gel at 15°C. For the enzyme elution, (a) a step gradient or (b) a linear (0–0.5 *M*) gradient of sodium chloride in 0.05 *M* MES (pH 5.5) was applied. X = Protein; enzyme activity (○) in the absence and (◇) in the presence of zinc ions.

TABLE I

EFFECT OF CHROMATOGRAPHY ON A METAL-FREE TED–SEPHAROSE COLUMN ON THE ZINC CONTENT AND ACTIVITY OF APEC

Each value is the average from 3–5 separate chromatographic experiments.

Chromatographic conditions for the enzyme elution	Moles of Zn^{2+} per monomer of enzyme	Activity		
		U/mg protein	%	% after incubation with Zn^{2+}
Control:				
Native enzyme	2.9	31.0	100	102.6
Enzyme after dialysis for 12 h against EDTA	0.5	6.1	19.6	63.7
Isocratic elution at 4°C	1.2	8.0	25.9	62.2
Elution by step gradient of NaCl at 15°C	1.2	3.7	12.1	46.0
Elution by linear gradient (0–0.5 *M*) NaCl at 15°C	1.1	3.3	8.1	19.6

of the properties of immobilized subunits of APEC showed that the catalytically inactive monomer of the enzyme binds tightly one zinc atom [10].

When APEC is chromatographed at 4°C, the enzyme does not bind to the gel. However, during chromatography at higher temperatures from 15 to 23°C (data for 23°C are not shown), APEC acquires the ability to bind with metal-free TED gel. The binding at higher temperature might be caused by an increase in binding energy, probably from electrostatic interactions of the gel itself or conformational changes of the enzyme. Alkaline phosphatase, as an oligomeric enzyme with an α/β topology of subunits, could undergo of molecular movements. Analysis of the topology indicated that binding crevices occur in the region where the strand order switches and that they are located at the carboxyl end of the strands. It was reported previously [11] that when the temperature is raised from 4°C to $\geqslant$20°C conformational changes of the enzyme are observed; for example, approximately four tyrosine residues per monomer are replaced on going from an aqueous to a hydrophobic environment. Therefore, the increase in accessibility of APEC binding to the metal-free chelated gel at higher temperature observed here, probably through zinc ions is not excluded.

Fig. 3 compares the effects of the chromatography on the activity of APEC and carboxypeptidase A (CPD). Passage of metalloenzymes through the metal-free TED gel reduces their catalytic activity. The decrease in activity is greatest when the eluting CPD has remained for some time (*ca.* 1 h) on a TED column. The delayed desorption phenomenon was first observed by Porath and Belew [4]. A new peak appeared when the elution of human serum from a Zn–TED–Sepharose 6B column was interrupted for 1 h or even for a shorter time. The desorbed peak differed substantially from the previously displaced proteins. Delayed desorption is not a phenomenon restricted to IMA gel but seems to reflect a property shared by all kinds of agarose gels and possibly cross-linked dextran and polyacrylamide gels [4].

The catalytic activity of chromatographed CPD is fully restored by the addition of Zn^{2+} ions, whereas the activity of APEC is restored to 75% of the initial activity

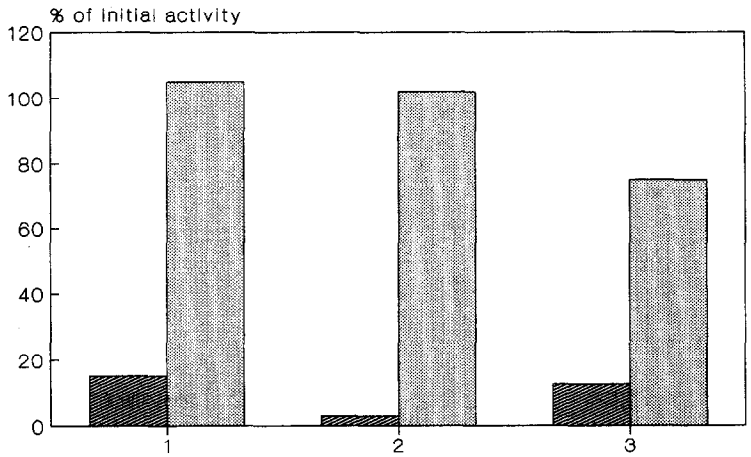


Fig. 3. Activities of metalloenzymes after elution from metal-free TED gel. Chromatography was performed at 4°C: (1) directly (carboxypeptidase); (2) with 1 h delayed desorption (carboxypeptidase); (3) with 1 h delayed desorption (alkaline phosphatase). Enzyme activity (hatched) without and (dotted) in the presence of zinc ions.

(Fig. 3). Similar results for APEC were obtained by using the solute chelating agent. Simpson and Vallee [12] found that when alkaline phosphatase was exposed to 8-hydroxyquinoline-5-sulphonic acid, two zinc atoms were rapidly removed and the enzyme was inactivated to *ca.* 10% of the initial level. When zinc was added to apoenzyme the activity was restored to 85% of the initial level. The differences in the restoration of the activity of the two enzymes might be a consequence of the different stabilities of the enzymes. Chromatography of the enzyme at higher temperature and dilution of the enzyme (*e.g.*, after elution caused by a linear gradient of salt, see Fig. 2b) have greater effects on the activity. In such a case reactivation of APEC by zinc ions might be less effective (Table I).

There is still some uncertainty concerning the role of zinc in the phosphatase activity [13]. The rate of inactivation of enzyme by EDTA (in the solution) seems to be biphasic, corresponding to different zinc binding sites associated with phosphatase [7]. Lazdunski *et al.* [7] found that after 50 min of EDTA treatment of alkaline phosphatase, zinc was partially removed from the enzyme, and prolonged exposure (12 h) of the enzyme to the chelator was able to remove essentially all of the metal from the protein. Their findings are consistent with our results. Short dialysis against EDTA (data not shown) does not have a further effect on the chromatographed enzyme, whereas prolonged dialysis causes a considerable decrease in zinc content. Prolonged dialysis is more effective than chromatography for removing the zinc from alkaline phosphatase.

Carboxypeptidase A is a much more stable enzyme than APEC. Carboxypeptidase A is a monomeric enzyme with one binding site for zinc ions; zinc ions are thought not to play a critical role in maintaining the overall three-dimensional structure of the enzyme. The metal was only essential for the catalytic activity of carboxypeptidase [14]. Although it has been shown that removing Zn^{2+} ions from APEC did not cause the dissociation of the dimer, the role of metal is probably more complex and cooperative.

Metalloenzymes offer unusual opportunities in the study of the mechanism of enzyme action. The physical and chemical properties of metal ions are readily differentiated from those of the amino acid side-chains of proteins, and thus become valuable probes of the active site. Therefore, the important task is the removal of metal ions from the proteins. For a long time chelating agents in solution have served this purpose. However, apart from the removal of the metal, free chelating agents may inhibit the enzymatic activity through the formation of mixed complexes. Also, the preparation of any apoenzyme in such a fashion includes a long procedure, mostly extensive dialysis, first against the chelator and second against buffer to remove the agent. The drawbacks to such an approach might be inactivation of the enzyme during prolonged dialysis and unpredictable effects of traces of chelating agents still present in the solution.

Therefore, the application of immobilized chelating agent for removing metal ions from proteins was highly desirable. Treatment of alkaline phosphatase with Chelex 100 resulted in a loss of the activity; the enzyme was reactivated by addition of zinc ions [15]. However, lack of data concerning the metal content of the enzyme without and after treatment with Chelex made the direct determination of the efficiency of the applied gel impossible.

Based on the determination of the catalytic activity, the effect of zinc on the reactivation and the measurement of metal content, we have now demonstrated the usefulness of metal-free TED gel for removing metal ions from proteins. However, it should be mentioned that the metal ion, which is very tightly bound, was not removed from proteins by this technique. Being conscious of some limitations, in our opinion the use of "reversed" IMAC, where the chelating agent is immobilized on the insoluble support, offers new possibilities for the characterization of metalloproteins and the preparation of their metal-free forms.

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