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PURIFICATION OF NUCLEOSIDEDIPHOSPHATASE OF RAT LIVER BY METAL-CHELATE AFFINITY CHROMATOGRAPHY

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Summary

A procedure is presented for the purification of nucleosidediphosphatase (nucleosidediphosphate phosphohydrolase, EC 3.6.1.6) of rat liver by affinity chromatography using metal conjugated to epoxy-activated Sepharose 6B. The enzyme is eluted from the conjugate by a solution of L-histidine. The enzyme, when bound to metal-chelate gel, is active in a suspended form, suggesting that the catalytic site is different from the site that binds to the metal-chelate gels. Substrate specificity and K_m value of the enzyme obtained are similar to those of the enzyme obtained from the same sources by a conventional procedure, indicating that the metal-chelate affinity chromatography does not bring about any substantial change in the catalytic properties.

Introduction

Porath [1] recently showed that chelate-forming ligands for metal ions (Zn^{2+} and Cu^{2+}) could be attached to biscarboxymethyl aminoagarose with the disodium salt of iminodiacetic acid and demonstrated that the affinity of proteins for the metal-chelate gel is pH dependent and the elution of proteins is achieved by reducing the pH and increasing the ionic strength.

Several reports have been published on the use of metal-chelate affinity chromatography for the purification of proteins [2—4].

Nucleosidediphosphatase (nucleosidediphosphate phosphohydrolase, EC 3.6.1.6) catalyzes the hydrolysis of nucleoside diphosphate to nucleoside

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monophosphate and inorganic phosphate [5—6] and has been studied extensively in terms of its catalytic and regulatory mechanisms [6—8]. Recently we have also shown the enzyme to be a metallo-enzyme containing zinc and manganese [9]. In the course of this study we found that nucleosidediphosphatase binds to the metal-chelate gel. This finding has made it possible to obtain a highly active enzyme by means of chromatography on metal-chelate Sepharose. The present study describes the first application of this method for the purification of metallo-enzymes and suggests that the elution of proteins by L-histidine from the gel is applicable to metal-chelate gel chromatography.

Materials and Methods

Chemicals. DEAE-cellulose was purchased from Whatman Ltd., Springfield, U.K. Sephadex G-200, PD-10 column and epoxy-activated Sepharose 6B were the products of Pharmacia Fine Chemicals A.B., Uppsala, Sweden. All other chemicals were of analytical grade.

Enzyme assay. Nucleosidediphosphatase activity was determined using 5 mM UDP as a substrate by the method described previously [8]. In this assay 1 unit of enzyme activity is defined as the amount of enzyme that releases 1 μmol of P_i per min from UDP, under the described conditions. Protein concentration was estimated by absorbance at 280 nm or by the method of Lowry et al. [10] with bovine serum albumin as a standard.

Partial purification steps of nucleosidediphosphatase. Nucleosidediphosphatase solubilized from rat liver microsomes (step 1) was subjected to $(NH_4)_2SO_4$ fractionation (40—70% saturation, step 2), DEAE-cellulose chromatography (step 3), and Sephadex G-200 chromatography (step 4) according to the method of Kuriyama [7]. The enzyme preparation at the step 4 (55 mg protein in 45 ml, 2613 units) was used for the metal-chelate affinity chromatography.

Metal-chelate affinity chromatography. The chelate-forming ligands: Preparation of the metal-chelate gels was essentially as described by Porath et al. [1]. Any oxirane groups remaining after coupling were blocked by incubation for 3 h with 1 M ethanolamine at 56°C.

Results and Discussion

Fig. 1 shows typical elution profile obtained when the partially purified enzyme (step 4, 55 mg protein, 2613 units) was applied to a column of copper-chelate (2 × 10 cm) chromatography. When the column was washed with maleate buffer (pH 6.6) containing 0.5 M NaCl, nucleosidediphosphatase activity was retained. The non-adsorbed protein was passed through with the wash. The column was subjected to elution with a linear gradient established between 150 ml of 10 mM maleate buffer (pH 6.6) and 150 ml of the same buffer containing 15 mM L-histidine. The concentration at 10 mM L-histidine successfully removed the enzyme from the column. The fractions containing nucleosidediphosphatase activity were pooled and dialyzed thoroughly against 20 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl. The dialyzate (10 mg protein, 1850 units) was applied on a zinc-chelate column (1 × 2 cm) previously equilibrated with the above buffer. Non-adsorbed protein was eluted by the same buf-

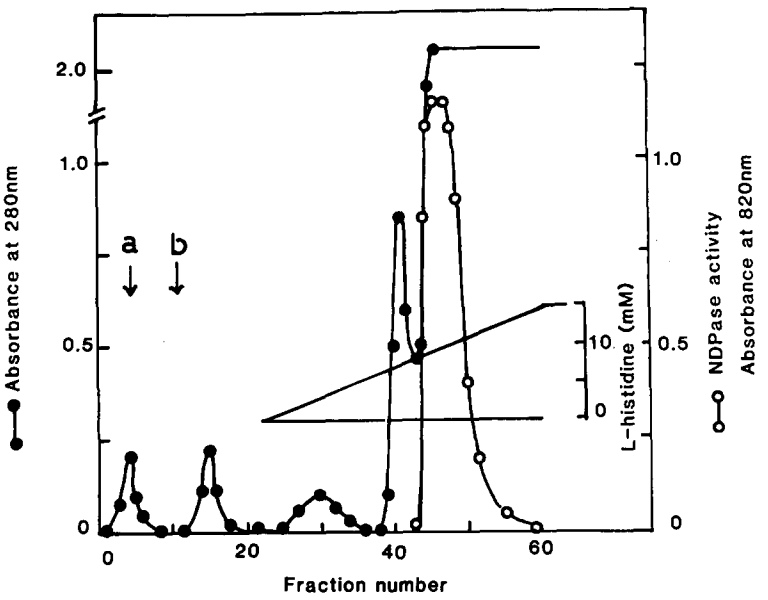

Fig. 1. Cu^{2+}-chelate affinity chromatography. Fractions of 'step 4' (obtained by Sephadex G-200, 55 mg protein in 45 ml) was applied on a column (2 X 10 cm) of Cu^{2+}-chelate affinity gel equilibrated with 20 mM Tris-HCl buffer (pH 7.5). The column was washed with (a) 100 ml of 10 mM maleate buffer (pH 6.6) at the point indicated by the arrow, and (b) 100 ml of the same buffer containing 0.5 M NaCl at the point indicated by the arrow, and then eluted by the linear gradient established between 150 ml of 10 mM maleate buffer (pH 6.6) and 150 ml of the same buffer containing 15 mM L-histidine at a flow rate of 20 ml/h. 3 ml fractions were collected and the protein and nucleosidediphosphatase (NDPase) activity were assayed as described in Materials and Methods. The active fractions were combined and subjected to the subsequent fractionations.

fer and the enzyme was eluted with 10 mM L-histidine in 10 mM maleate buffer (pH 6.6) as shown in Fig. 2. The recovery of nucleosidediphosphatase was 867 units in 2.8 mg protein. The yield was 47 and 15.5% of the applied activity at step 4 and step 5, respectively. In a separate experiment, attempts to recover the protein from the copper- (or zinc-) column by elution with 10 mM EDTA were also effective. However an attempt to elute the enzyme by reducing pH brought about the inactivation of the enzyme and was abandoned. No elution of nucleosidiphosphatase could be achieved with the following solution; 1 M NaCl, 0.1 M Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl to 0.8 M NaCl, 10 mM udp, 10 mM GTP, 1% deoxycholate or 1 mM cysteine in 10 mM maleate buffer (pH 6.6). Yields in the purification steps of the liver enzyme including procedure are summarized in Table I. The specific activity of the purified enzyme is of the same order as that obtained by the method of Kuriyama [7]. However, complete purification should be possible by use of an additional step. The purified enzyme obtained by affinity chromatography had a K_m value of 2 mM for UDP, almost identical to that obtained by a conventional technique. No significant changes in the substrate specificity for UDP, IDP and GTP were observed between the two enzymes (data not shown).

When the zinc- and copper-chelate gels bound nucleosidediphosphatase were removed from the column and suspended in buffer and assayed with UDP as the substrate under the standard conditions, significant activity (almost full

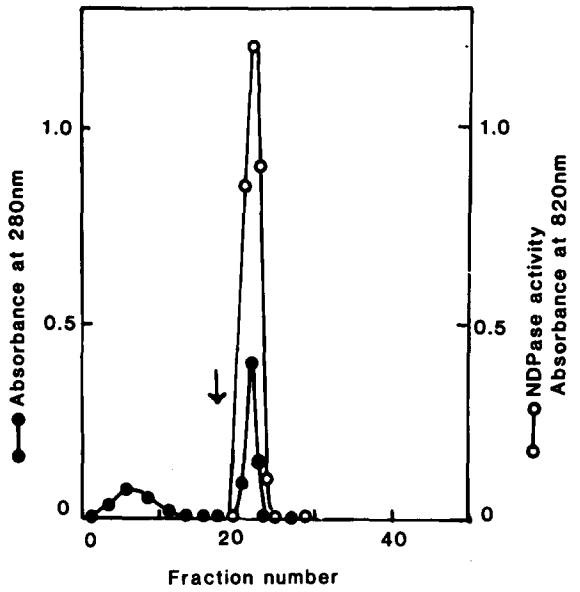

Fig. 2. Zn^{2+}-chelate affinity chromatography. Fractions of 'step 5' (10 mg protein in 35 ml) were applied on a column (1 × 2 cm) of Zn^{2+}-chelate affinity gel equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl. After non-adsorbed proteins flowed out with 15 ml of the same buffer and then the elution was made with 15 ml of 10 mM L-histidine in 10 mM maleate buffer (pH 6.6) at the point indicated by the arrow at a flow rate of 15 ml/h. 1 ml fraction was collected and the protein and nucleosidediphosphatase (NDPase) activity were assayed as described in Materials and Methods.

activity) could be recovered. In fact the solid phase became blue in zinc-chelate gel (the gel is originally white in color) and much bluer in the copper-chelate gel (the gel is originally light blue), showing release of inorganic phosphate from their gel surface.

This activity could be destroyed by prior heating of the materials at 100°C. This observation is consistent with the fact that neither UDP nor GTP was able to elute the bound enzyme from the metal conjugate. Thus these results could indicate that the binding site of the nucleosidediphosphatase which had affinity for the conjugated gel is apparently different from the active site in nucleo-

TABLE I

PURIFICATION OF NUCLEOSIDEDIPHOSPHATASE

Step	Total volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification (-fold)
1. Solubilized supernatant	500	3500	5600	1.6	100	1
2. $(NH_4)_2SO_4$ (40—70%)	105	1365	5050	3.7	90	2.3
3. DEAE-cellulose	155	171	3505	20.5	63	12.8
4. Sephadex G-200	45	55	2613	47.5	47	29.7
5. Cu^{2+}-column	35	10	1850	185	33	116
6. Zn^{2+}-column	3	2.8	867	310	15.5	194

sidediphosphatase. It would appear that the binding of the enzyme to the conjugated gel is mediated by the histidine residues in nucleosidiphosphatase molecule, since excess of L-histidine or EDTA resulted in successful elution. As described by Porath et al. [1], imidazole and thiol groups may interact with Zn^{2+} or Cu^{2+} fixed on the gel but it is still not possible to draw a parallel relation between exposed histidine and cysteine (or other amino acids) reidues in the protein surface. Most of the zinc-containing enzymes have histidine or cysteine residues in the active sites [11]. The present study indicated that the site which binds to the metal-chelate gel is different from the catalytic site of the enzyme. In our recent studies on the catalytic mechanism of nucleosidediphosphatase we found that the enzyme has a structural site and activation site by metal ions [9]. The present and previous results suggested that the metal-chelate gel binds to a structural site, which is possibly composed of histidine residues in a coordination complex with Zn^{2+} and Cu^{2+}. The present study also suggested that some other metallo-enzyme or metal dependent enzyme may have an affinity for the metal-chelate gel and metal-chelate affinity chromatography is applicable for the purification of the metalloenzyme.

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