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IMMOBILIZED METAL ION AFFINITY CHROMATOGRAPHY IN ENZYME FRACTIONATION

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Ribitol dehydrogenase from *Mycobacterium butyricum* and α-mannosidase from *Lupinus luteus* seedlings were fractionated by the immobilized metal ion (Cu^{2+} or Zn^{2+}) affinity chromatography (IMAC) on iminodiacetic acid coupled to Sepharose 6B. In a single step, ribitol dehydrogenase was purified 10-12 fold with the recovery above 80% when using Zn^{2+}-Sepharose 6B as the sorbent and decreasing linear gradient of pH from 7 to 4. In the same conditions purification of α-mannosidase was less effective (2-3 fold, recovery 60-70%).

Since its introduction by Porath's group [1], the immobilized metal ion affinity chromatography (IMAC) has been used as an efficient method for purification of a wide variety of proteins and peptides [2-5]. In this method the advantage is made of the ability of the ions (i.e. Cu, Zn, Co, Ni) to form stable coordinated complexes with reactive imidazoyl (histidine) and cysteinyl residues on the surface of proteins [1].

The most popular chelating ligand, used by the Porath's group [1], is iminodiacetic acid (IDA) coupled to agarose *via* oxirane spacer. IDA-Sepharose 6B forms stable but reversible complexes with metals and is suitable for purification procedures. The recently introduced TED-Sepharose 6B [tris(carboxymethyl)ethylenediamine-Sepharose 6B] binds metal ions very tightly [6]. Thus, the metal-free TED-Sepharose 6B a good tool in "reversed" IMAC, designed to "withdraw" metal components from metalloprotein structures [7]. Besides, IMAC is well suited for h.p.l.c. (HP-IMAC [8]). These methods may serve as facile probes of the topography of reactive groups of proteins [3, 9] and also for immobilization of enzymes [10]. In this report, we applied IMAC for fractionation of ribitol dehydrogenase of bacterial origin and plant α-mannosidase.

MATERIALS AND METHODS

Ribitol dehydrogenase (RDH). The enzyme was prepared from *Mycobacterium butyricum*, grown for 4-5 days at 37°C on liquid glucose--glutamate-citrate-salts medium (pH 6.7) in Roux flasks [11]. The bacteria

(70 g) were sonicated and the enzyme was salted out from the cell-free extract [12] at 0.35-0.70 saturation. The contaminating proteins were coagulated by heating at 70°C for 1 min, and the dialyzed supernatant was applied on DEAE-cellulose DE-53 column (2.2 × 25 cm). The enzyme was eluted with a linear NaCl 0-0.5 M gradient. The pooled active fractions were concentrated by ultrafiltration, dialyzed and used for IMAC.

α-Mannosidase was prepared from Lupinus luteus seedlings. The enzyme from the cell-free extract [13] was purified by the same procedure as used for ribitol dehydrogenase.

Ribitol dehydrogenase was assayed spectrophotometrically at 340 nm using meso-erythritol and NAD [12] and α-mannosidase with *p*-nitrophenyl-α-D-mannopyranoside as a substrate [13].

Protein content was determined by the method of Lowry *et al.* [14].

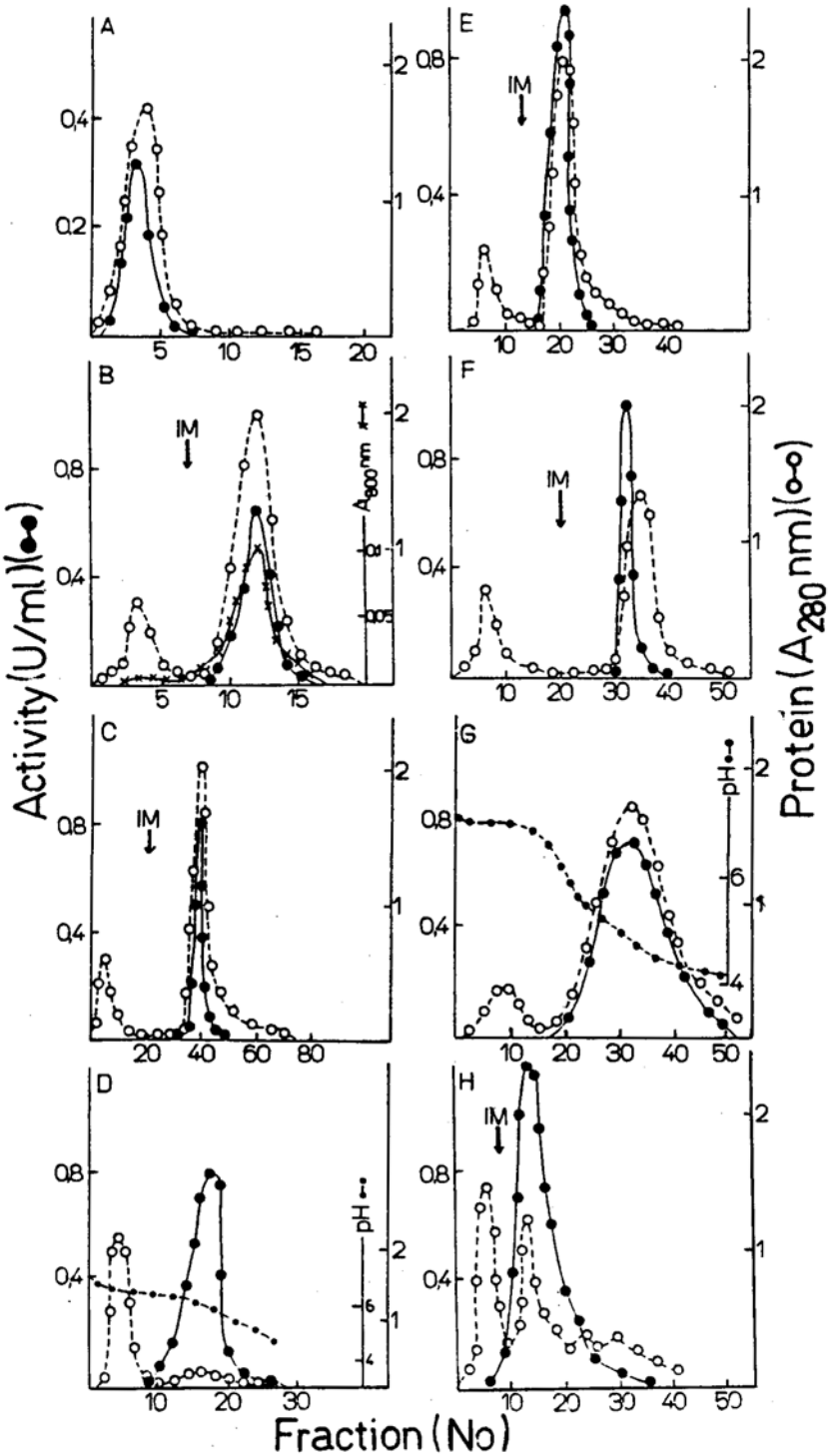
Chelating Sepharose column. The column was prepared using IDA-Sepharose 6B (Pharmacia, Sweden) gel suspended in water. The column was loaded either with the solution of $CuSO_4 \times 5H_2O$ (5 mg/ml^{-1}) until 2/3 volume of the gel was coloured blue, or with the solution of $ZnSO_2 \times 7H_2O$ (5 mg/ml^{-1}) until Zn^{2+} ions appeared in the eluate. Then, the column was thoroughly washed with 25 mM phosphate buffer, pH 7.0, containing 1 M NaCl. The columns were regenerated by removing metal ions and the firmly bound material with 50 mM EDTA.

RESULTS

In our previous report [12] we have suggested that mycobacterial ribitol dehydrogenase (RDH) might be another new zinc polyol dehydrogenase distinct from the already known zinc-containing sorbitol dehydrogenase [15]. However, the possible metalloprotein nature of RDH can only be elucidated by isolation of the enzyme in homogeneous form and measuring both its activity and zinc content.

Figure 1 (left side) shows a set of fractionation profiles of partially purified RDH preparations on IDA-Sepharose 6B column in different chromatographic conditions. Total RDH passed through the metal-free column (Fig. 1A) whereas it was completely retained on the metal-loaded gel. Thus, the retention is evidently connected with the formation of stable chelates between metal ions on the matrix and the reactive groups of the enzyme.

Fig. 1. Immobilized metal ion affinity chromatography (IMAC) of ribitol dehydrogenase from *M. butyricum* (left) and α-mannosidase from *L. lupinus* seedlings (right). DEAE-cellulose eluates (5 ml, 10-25 mg protein) were used for fractionations. The column (1 × 21.7 cm) was metal-free (A), loaded with Cu^{2+} (B, C, E, F) or Zn^{2+} ions (D, G, H). The elution was performed with 50 mM phosphate buffer containing 1 M NaCl (A), 50 mM imidazole (IM) (B, E), linear imidazole gradient of 0-50 mM (C, H) or pH gradient 7-4 (D, G). A_{800} nm denotes concentration of Cu^{2+} ions in the eluates


A
B
C
D
E
F
G
H
IM
Activity (U/ml)(●-●)
Protein ($A_{280\,nm}$)(○-○)
$A_{800\,nm}$
pH
Fraction (No)

Cu^{2+} - IDA - Sepharose 6B binds RDH and some accompanying proteins very efficiently, yet no selective enzyme elution could be obtained as one can see when either step - wise (Fig. 1B) or linear (Fig. 1C) gradients of a competing solute, imidazole, were introduced. The same was also true with a linear pH gradient (not shown). Besides, two other disadvantages appeared in the course of fractionations. The first was a leakage of Cu^{2+} ions from the column as shown spectrophotometrically at 800 nm (Fig. 1B). Cu^{2+} ions inactivated RDH very rapidly ($t_{1/2}$ 5 min at 3 mM Cu^{2+}). Another disadvantage was the inhibition of RDH by imidazole (50% inhibition at 15 mM imidazole) (Fig. 2).

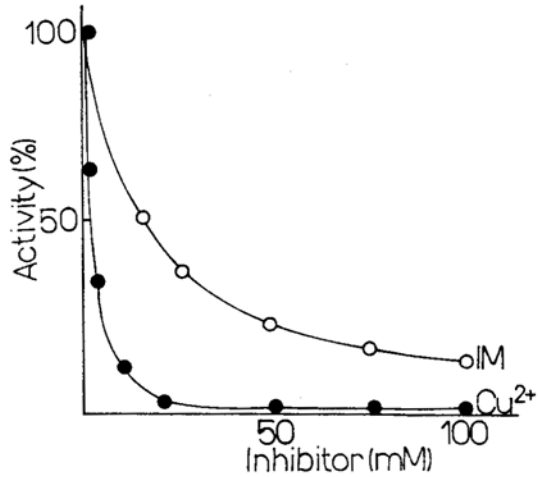


Fig. 2. Effect of imidazole (IM, ○) and Cu^{2+} ions (●) on ribitol dehydrogenase. The enzyme was preincubated with the inhibitors for 5 min. Assays were performed as presented in Methods

In comparison to Cu^{2+} - IDA, Zn^{2+} - IDA - Sepharose 6B column was fully successful for RDH purification. As can be seen (Fig. 1D) most of the inactive proteins passed through the column when a decreasing linear gradient of pH from 7 to 4 was applied and the enzyme was eluted in the range of pH 6.5 - 5.5. In a single step, 10 - 12 fold purification of RDH with more than 80% recovery was achieved.

The other enzyme studied was a cytosolic α - mannosidase from lupine seedlings. Like RDH, α - mannosidase was not adsorbed on the metal - free IDA - Sepharose 6B (not shown) but on the column with Cu^{2+} (Fig. 1E, F) or Zn^{2+} (Fig. 1G, H) ions, the enzyme was fully retained together with a bulk of inactive proteins. Here, as well, the enzyme could be eluted with the use of imidazole solution (Fig. 1E, F, H) or acidic buffer (Fig. 1G). However, the results were not as good as with RDH (Fig. 1D) since only 2 - 3 fold purification with 60 - 70% recovery was obtained. Nevertheless, this method could be used in the standard α - mannosidase purification procedure.

In conclusion, IMAC methods can be useful in enzyme fractionation provided suitable metal chelates and a proper eluent are used.

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