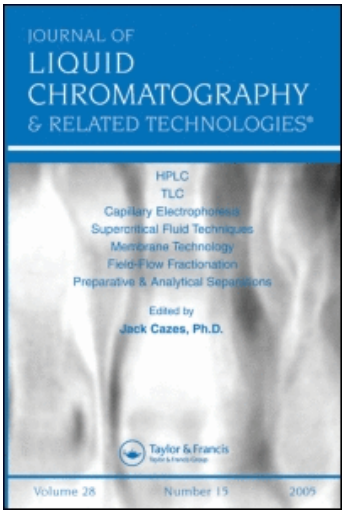

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AFFINITY CHROMATOGRAPHY OF RIBITOL DEHYDROGENASE, A POSSIBLE NEW ZINC ENZYME FROM MYCOBACTERIUM BUTYRICUM

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ABSTRACT

An effective purification procedure of ribitol dehydrogenase (RDH), a possible new zinc enzyme from Mycobacterium butyricum is described. The procedure took advantage of different chromatographic methods in which the most significant were two affinity chromatography steps. One of them was the immobilized metal ion affinity chromatography (IMAC), with the use of imino-diacetate-Sepharose 6B (IDA-Sepharose 6B) chelating Zn^{2+} ions (IDA-Zn) as an affinity sorbent. The enzyme was eluted with a decreasing pH gradient from 7 to 4. The other step was a biospecific affinity chromatography, where the enzyme retained on 5'AMP-Sepharose 6B was eluted with 10 mM adenosine 5'-monophosphate (AMP). RDH was purified 174-fold with 10.2% of recovery, and the final preparation was homogenous in polyacrylamide gel electrophoresis.

INTRODUCTION

Among the several polyhydric alcohol dehydrogenases described in nature, sorbitol dehydrogenase is, to the best of our knowledge, the only enzyme of this type which

contains zinc in the structure (1,2). However, we showed in mycobacteria the occurrence of ribitol dehydrogenase which revealed the absolute requirements for Zn^{2+} ions in the growth medium (3). The enzyme was also sensitive to chelating agents (4). These indirect proofs indicate that ribitol dehydrogenase (RDH) from mycobacteria might be a new kind of zinc enzyme. However, the possible metalloprotein nature of RDH can only be proven by isolating the homogenous enzyme from the cells and measuring both activity and zinc.

In this report, we chose Mycobacterium butyricum as a good source of RDH and used a biospecific affinity chromatography, an immobilized metal ion affinity chromatography as well as other chromatographic methods for isolation of the homogenous protein.

MATERIALS AND METHODS

Special reagents.

Meso-erythritol was from Koch-Light Lab. Ltd (Colnbrook, England); adenosine 5'-monophosphate (AMP) - Serva (Heidelberg, Germany); nicotinamide adenine dinucleotide (NAD) - Reanal (Budapest, Hungary); Tris(hydroxymethyl)aminomethane - Sigma Chemical Co (St.Louis, USA); DEAE-cellulose DE-53 - Whatman Biochemical Ltd (Meidstone, England); 5'AMP-Sepharose 6B, Chelating Sepharose, Sephadex G-200 and Sephacryl S-300 were from Pharmacia (Uppsala, Sweden).

Buffers.

Buffer A, 50 mM phosphate buffer (pH 7.3); Buffer B, 10 mM phosphate buffer (pH 7.3); Buffer C, citrate/phosphate buffer (pH 7.0) containing 1 M sodium chloride To all buffers 2.5 mM 2-mercaptoethanol was included.

Chromatographic columns.

Sephacryl S-300 column (2.2 x 84 cm) and Sephadex G-200 column (2.5 x 100 cm) were equilibrated with Buffer A. DEAE-cellulose DE-53 column (2.2 x 25 cm) and 5'AMP-Sepharose 6B column (1 x 15 cm) were equilibrated with Buffer B. Chelating Sepharose (Iminodiacetate-Sepharose 6B) charged with Zn^{2+} ions (IDA-Zn) column (1 x 22 cm) was equilibrated with Buffer C.

Fractionation procedure.

M. butyricum cells (60 g) were grown, harvested, disrupted in an ultrasonic disintegrator and centrifuged as previously described (5). The resulting cell-free extract was fractionated with ammonium sulfate, and the proteins precipitated between 0.4 and 0.7 saturation were collected by centrifugation. These were dissolved in 40 ml of Buffer A. Portions of 2 ml of the aliquot were heated for 1 min at 70°C, cooled and the coagulated proteins were discarded by centrifugation. The supernatant was dialyzed overnight against Buffer B (Ash-fraction).

The Ash-fraction was applied on DEAE-cellulose column, washed with Buffer B and enzyme was eluted with a linear gradient of NaCl 0-0.5 M (250 ml in each vessel). The pooled active fractions were concentrated to about

4 ml by ultrafiltration and put on a Sephacryl S-300 column. Elution was performed with Buffer A. The active fractions were concentrated again to the same volume and applied on IDA-Zn column. This was washed with Buffer C, and the enzyme was eluted with a decreasing linear gradient of pH 7-4 (100 ml). The active material was concentrated to 3 ml and fractionated next on a Sephadex G-200 column. The final step was the chromatography on 5'AMP-Sepharose 6B column. The concentrated active fractions from the preceding step (4 ml) were made 0.5 M with NaCl and put on the column. This was washed with Buffer B, followed by 0.5 M NaCl and Buffer B. The enzyme was eluted with 10 mM AMP. The concentrated final preparation (2 ml) was dialyzed and underwent polyacrylamide gel electrophoresis to check the purity.

Enzyme assay.

The enzyme was assayed with a standard system which contained 120 μmol glycine/NaOH buffer (pH 9.7), 40 μmol erythritol, 0.5 μmol NAD and enzyme in the total volume of 1.5 ml. Increase in absorbance at 340 nm at room temperature followed for 3 min. One unit of activity is defined as the amount of enzyme which catalyzed the reduction of 1 μmol NAD per min under the specified conditions. Proteins were measured with the Folin reagent (6).

Polyacrylamide gel electrophoresis.

The method of Davis (7) was used. The proteins were stained with Amido Black. Activity staining was performed by incubating the gel (37°C, 30 min) in the assay mixture

also containing 0.1 ml nitrotetrazolium blue (8 mg/ml) and phenazine methosulfate (4 mg/ml).

RESULTS AND DISCUSSION

In this proposed RDH purification procedure the most effective were two affinity chromatography steps. One of them was the immobilized ion affinity chromatography (IMAC), a method first introduced by Porath (8) for selective separation of proteins. IMAC exploits the ability of transition elements (i.e. Cu, Zn, Ni, Co) to form stable coordination complexes with electron -rich amino acid residues, such as histidine, cysteine and tryptophan (Porath's triade), which are located on the surface of proteins (9,10). These elements are attached to a solid matrix via immobilized ligands, mostly imminodiacetic acid (IDA), producing a polydentate chelates. Owing to this method, different proteins were separated and purified, as for instance human growth hormone (11), tissue plasminogen activator (12), glycophorins (13), carboxypeptidase B (14) and α-amylases (15). For more examples readers are referred to Lönnerdal and Keen (16) and Sulkowski (17). A number of other applications of IMAC encompassing separation of cells, immobilization of enzymes, HPLC separation of peptides and hormones and others, have been reviewed by Arnold (18). A new approach defined as "reversed" IMAC can be applied to withdraw a metal component from the structure of metalloproteins, but in these case tris(carboxyme-

thyl)ethylenediamine (TED) is preferable to IDA (19). The above-mentioned examples demonstrate the versatility of IMAC which is still less popular chromatographic method than it should be.

When comparing the adsorption capacity of different immobilized metal chelates to serum protein, Porath's group (8) found in their pioneer study the following order: Cu>Zn>Ni>Mn. Thereafter IDA-Cu has become the most frequently used chelate in IMAC, and IDA-Zn has been next in importance. Unfortunately, our attempts to apply the immobilized IDA-Cu for the purification of RDH were in failure. While the enzyme was fully retained on the column, yet its desorption was completely non-selective process, in spite of different eluants used (linear or step-wise gradients of imidazol, or pH). Furthermore, a continuous leakage of cooper ions from the matrix inactivated the enzyme rapidly (half-life of RDH at 3 mM Cu^{2+} ions was found to be 5 min). However, IDA-Zn appeared to be an excellent adsorption material. For instance, in one fractionation of the DEAE-cellulose elu - ate, when using a pH gradient elution (from 7 to 4), 11-fold purification of RDH with 84% of recovery was obtained. Therefore, this method was applied in the standard purification procedure (Fig. 2), after DEAE-cellulose (Fig. 1) and Sephacryl S-300 steps. Noteworhy is that imidazol, the compound widely used in IMAC, cannot be applied for the elution of RDH since it has an inhibitory activity towards the enzyme (not shown).

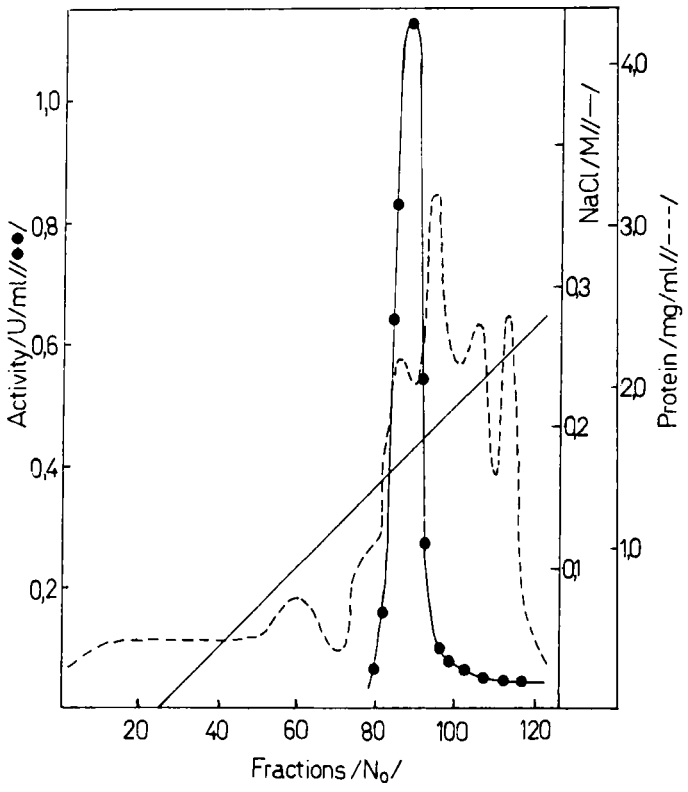

Figure 1: Ion-exchange chromatography of ribitol dehydrogenase from M. butyricum on DEAE-cellulose DE-53 column. Ash-fraction (43 ml, 48.6 units) was applied on the column (2.5 x 25 cm). Elution was performed with the linear gradient of NaCl from 0 to 0.5 M (250 ml in each vessel), and 4 ml-fractions were collected.

A biospecific affinity chromatography was the other step succesfully used in RDH purification. It is well-known in case of NAD-dependent dehydrogenases that AMP and NAD serve as "general ligands", while NAD or its reduced form - NADH are used as eluting factors (20). In

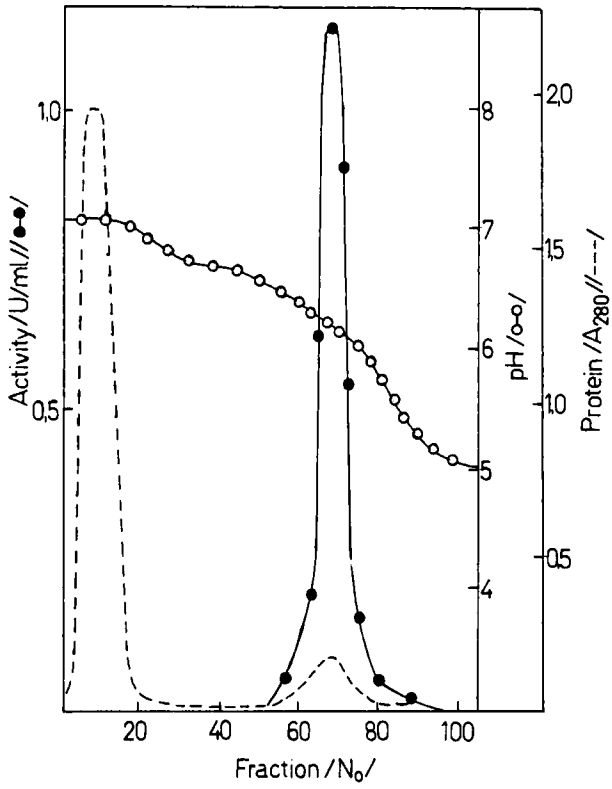

Figure 2: Immobilized metal ion affinity chromatography of ribitol dehydrogenase on IDA-Zn column. Sephacryl S-300 eluate (4 ml, 19.2 units) was applied on the column (1 x 22 cm). Elution was performed with the linear pH gradient of Buffer C from 7 to 4 (100 ml in each vessel), and 2 ml-fractions were collected.

our study we attempted the use of 5'AMP-Sepharose 6B as the affinity adsorbent and AMP as the much cheaper competitive ligand for the elution of the RDH (Fig. 3).

Table 1 shows the purification and recovery of the 4 parallel fractionation procedures in which the enzyme was

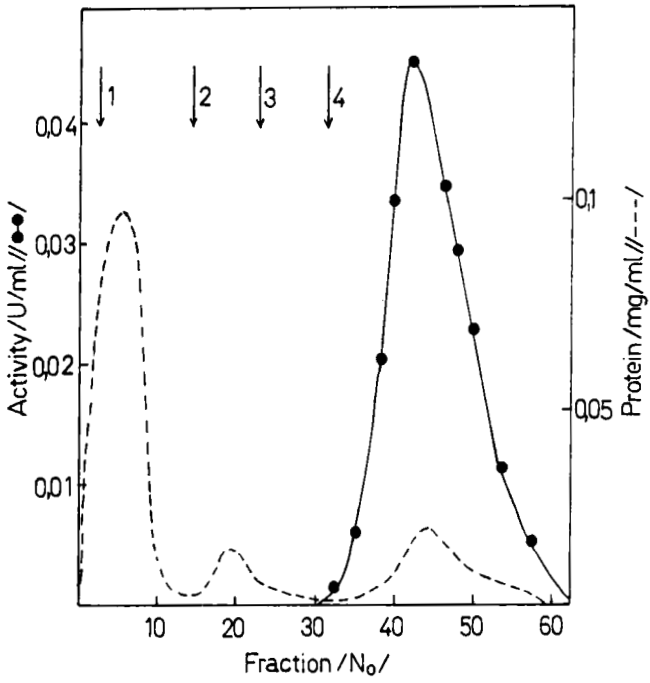

Figure 3: Biospecific affinity chromatography of ribitol dehydrogenase on 5'AMP-Sepharose 6B column. Sephadex G-200 eluate (4 ml, 14.4 units) were applied on the column (1 x 15 cm). Elution was performed with 10 mM AMP, and fractions of 4 ml were collected. 1,3 Buffer B; 2, 0.5 M NaCl; 4, 10 mM AMP.

eluted from 5'AMP-Sepharose 6B column with increasing concentrations of AMP. One can see that 10 mM AMP is preferable since in this case RDH was purified 10-fold with 80% of recovery. Higher concentrations of AMP should rather be avoided because of the competitive inhibition of RDH (data not shown). Other compounds tested, namely: erythritol (50 mM), KCl (1 M), ADP (10 mM) were very poor desorption factors.

Table 1

Effect of AMP Concentration on the Desorption of Ribitol Dehydrogenase from the 5'AMP-Sepharose 6B column

AMP (mM)	Protein (mg)	Activity total (units)	Activity spec. (U/mg)	Purification	Yield (%)
		Applied			
	14.0	3.1	0.22		100
		Recovered			
1	0.09	0	0	0	0
5	0.23	0.91	3.95	17.9	29.3
10	0.24	2.5	2.27	10.3	80.6
15	0.19	1.45	1.66	7.5	46.7

1 ml-portions of the Ash-fraction (3.1 units) were applied on the 5'AMP-Sepharose 6B column (1 x 15 cm). Protein denotes proteins eluted by AMP.

The entire RDH purification procedure can be summarized as follows. We processed a cell-free extract (300 ml) having 79.5 units of the total activity and 0.034 units/mg protein specific activity. As a result, we obtained a final preparation possessing respectively 8.12 units and 5.94 units/mg. This makes 174-fold purification of RDH with 10.2% of recovery. The preparation showed one protein band in the polyacrylamide gel electrophoresis that coincided strictly with the band of the enzyme activity.

REFERENCES

1. Jörnvall, H., Bahr-Lindström, H., Jany, K.D., Ulmer, W. and Froschle, M., Extended Superfamily of Short Alcohol-Sugar Dehydrogenases: Structural Similarities Between Glucose and Ribitol Dehydrogenases. FEBS Lett., 165, 190, 1984.

2. Jörnvall, H., Persson, B. and Jeffery, J., Characteristics of Alcohol-Polyol Dehydrogenases. The Zinc Containing Long-Chain Alcohol Dehydrogenases. FEBS Lett., 167, 185, 1987.

3. Szumiło, T., Szymona, O. and Szymona, M., Separation and Properties of Polyhydric Alcohol Dehydrogenases from Mycobacterium sp. 279 and Mycobacterium phlei. Acta Microbiol. Polon., 29, 332, 1980.

4. Szumilo, T. and Byra, A., The Possible Occurence of Zinc in Ribitol and Sorbitol Dehydrogenases from Mycobacterium sp. 279. Acta Biochim. Polon., 31, 401, 1884.

5. Szumilo, T., A Novel Enzyme, Tagatose Kinase, from Mycobacterium butyricum. Biochim. Biophys. Acta, 660, 366, 1981.

6. Lowry, O., Rosebrough, N.J., Farr, A.L. and Randall, R.V., Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem., 193, 265, 1951.

7. Davies, G., Disc Electrophoresis. II Method and Application in Human Serum Protein. Ann. N. Y. Acad. Sci., 121, 404, 1964.

8. Porath, J., Carlsson, J., Olsson, J, and Belfrage, G., Metal Chelate Affinity Chromatography, a New Approach to Protein Fractionation. Nature, 258, 598, 1975.

9. Porath. J. and Olin, B., Immobilized Metal Ion Affinity Adsorption and Immobilized Metal Ion Affinity Chromatography of Biomaterials. Serum Protein Affinities for Gel-immobilized Iron and Nickel. Biochemistry, 22, 1628, 1983.

10. Sulkowski, E., Immobilized Metal Ion Affinity Chromatography of Proteins. In: Protein Purification: Micro to Macro, Burges, R. ed., Alan R. Liss, New York, 1987, p. 149.

11. Maisano, F., Testpro, S. and Grandi, G., Immobilized Metal Ion Affinity Chromatography of Human Growth Hormone. J. Chromatogr., 472, 422, 1989.

12. Furlong, A.M., Thomsen, D.R., Marotti, K.R., Post, L.E. and Sharma, S.K., Active Tissue Plasminogen Activator Secreted from Insect Cells Using a Baculovirus Vector. Biotech. and Appl. Biochem., 10, 454, 1988.

13. Corradini, D., Rassi, Z.E., Horvath, C., Guerra, G. and Horne, W., Combined Lectin-Affinity and Metal-Interaction Chromatography for the Separation of Glycophorins by High-Performace Liquid Chromatography. J. Chromatogr., 458, 1, 1988.

14. Hortin, G.L. and Gibson, B.L., Purification of Carboxypeptidase B by Zinc Chelate Chromatography. Preparative Biochem., 19, 49, 1989.

15. Zawistowska, U., Saugster, K., Zawistowski, J., Langstaff, J. and Friesen, A.D., Immobilized Metal Ion Affinity Chromatography of Wheat α-Amylases. Cereal Chemistry. 65, 413, 1988.

16. Lönnerdal, B. and Keen, C.L., Metal Chelate Affinity Chromatography of Proteins. J. Appl. Biochem., 4, 203, 1982.

17. Sulkowski, E., Purification of Proteins by IMAC. Trends Biotechnol., 3, 1, 1985.

18. Arnold, F., Metal Affinity Separations: a New Dimenssion in Protein Processing. Biotechnology, 9, 151, 1991.

19. Muszyńska, G., Zhao, Y-J. and Porath, J., Carboxypeptidase A: a Model for Studying the Interaction of Proteins with Immobilized Metal Ions. J. Inorg. Biochem., 26, 127, 1986.

20. Mosbach, K., AMP and NAD as "General Ligands". Methods Enzymol., 34, 229, 1974.