PURIFICATION OF ALCOHOL DEHYDROGENASE FROM *BACILLUS STEAROTHERMOPHILUS* BY AFFINITY CHROMATOGRAPHY

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1. Introduction

Many dehydrogenases bind NAD^+ as coenzyme. This property has already been used in the preparation of glyceraldehyde-3-phosphate dehydrogenase from two thermophilic bacteria, *Bacillus stearothermophilus* and *Thermus aquaticus,* by affinity chromatography on NAD-Sepharose, made according to the method of Hocking and Harris [1]. We describe here a similar, large scale purification for alcohol dehydrogenase from a partly purified preparation from *B. stearothermophilus* (A. Atkinson, G. T. Banks, C. J. Bruton, M. J. Comer, R. Jakes, A. Kamalagharan, A. R. Whitaker and G. P. Winter, manuscript in preparation). This is a rapid, one step method, whereas the method previously determined for this enzyme by Kolb and Harris [2] involved four steps with the ultimate production of very poor yields of pure enzyme.

2. Materials and methods

B. stearothermophilus cells were grown, and the initial purification steps carried out at the Microbiological Research Establishment, Porton Down, Wiltshire, England (A. Atkinson, G. T. Banks, C. J. Bruton, M. J. Comer, R. James, A. Kamalagharan, A. R. Whitaker and G. P. Winter, manuscript in preparation). An 'ADH-rich' fraction containing 32 units/ml (specific activity 4.2 units/mg) alcohol dehydrogenase activity was prepared as part of a multienzyme preparation and stored in 50% glycerol at −20°C.

This fraction was applied directly to a column (40 × 3 cm) of NAD-Sepharose [1] equilibrated in buffer A (100 mM sodium phosphate pH 6.8, 3 mM 2-mercaptoethanol, 1 mM EDTA). The resin was washed with buffer A containing 700 mM sodium chloride until all protein with low affinity had been removed. Alcohol dehydrogenase was then recovered from the column in 400 ml buffer A containing 500 mM sodium chloride, 10 mM NAD (Sigma AA grade). The eluant was pooled and dialysed against three changes of 10 litres of buffer containing 20 mM sodium phosphate pH 6.8, 3 mM 2-mercaptoethanol, 1 mM EDTA to remove excess NAD.

Alcohol dehydrogenase activity was followed by the method of Racker [3]. Purity of the enzyme was established by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulphate [4] and by N-terminal analysis by the dansyl method [5]. Protein concentration was estimated by the method of Lowry et al. [6]. Amino acid composition was determined following hydrolysis of the protein in 6 N hydrochloric acid containing 1% phenol under vacuum at 105°C and subsequent analysis on a Durrum D-500 amino acid analyser.

3. Results and discussion

Affinity chromatography on NAD-Sepharose has made it possible to purify large amounts of alcohol dehydrogenase from *B. stearothermophilus* in one

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rapid step from an ADH-rich fraction. Enzyme (500 mg) with a specific activity of 13.1 units/mg was purified on 300 ml settled bed volume of NAD-Sepharose resin. The material eluted from the resin accounted for 75% of that absorbed and gave a single band when examined by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulphate. Figure 1 shows the purity of the ADH fraction before and after NAD-Sepharose chromatography. The pure enzyme gave a single N-terminal residue, methionine, by the dansyl method [5] and has the amino acid composition shown in table 1. Alcohol dehydrogenase from *B. stearothermophilus* is a tetramer with a

Fig.1. SDS-gel electrophoresis [4] on a 12.5% polyacrylamide gel of fractions containing *B. stearothermophilus* alcohol dehydrogenase (a) before and (b) after affinity chromatography on NAD-Sepharose.

Table 1

Amino acid	No. of residues	
Cm-Cysteine[a]	5.4	(5)
Aspartic acid	24.2	(24)
Threonine	12.6	(13)
Serine	10.3	(10)
Glutamic acid	33.7	(34)
Proline	16.4	(16)
Glycine	31.6	(32)
Alanine	32.4	(32)
Valine	39.5	(40)
Methionine	4.5	(5)
Isoleucine	17.8	(18)
Leucine	22.3	(22)
Tyrosine	11.3	(11)
Phenylalanine	8.7	(9)
Histidine	8.8	(9)
Lysine	21.4	(21)
Tryptophan[b]		
Arginine	11.8	(12)
Total		314

[a] Cm-Cysteine – carboxymethyl cysteine was estimated from carboxymethylated alcohol dehydrogenase

[b] The presence of tryptophan was shown by the staining method of Spies and Chambers [10]

molecular weight of 135 000 [2] and although smaller than the yeast enzyme, a tetramer of 150 000 molecular weight [7], the two enzymes show marked similarity in amino acid composition and sequence around the active site (ref. 8 and C. Woenckhaus, R. Jeck, J. I. Harris and M. J. Runswick, manuscript in preparation). Glyceraldehyde-3-phosphate dehydrogenase from the same bacterium is a thermostable, tetrameric, enzyme which binds four molecules of its coenzyme, NAD. Because of its thermal stability it has been possible to crystallise the enzyme with a number of NAD stoichiometries from nought to four moles per tetramer [9]. Alcohol dehydrogenase from *B. stearothermophilus* may be used to make similar structural studies.

The thermal stability of the enzyme has been of advantage in studies on its modification by NAD analogues (C. Woenckhaus, R. Jeck, J. I. Harris and M. J. Runswick, manuscript in preparation). These experiments further show the similarity between yeast and *B. stearothermophilus* alcohol dehydrogenase, where the same cysteine residue is modified, but a

dissimilarity with the enzyme from horse liver, where a different cysteine, not involved in the active site, is modified.

References

[1] Hocking, J. D. and Harris, J. I. (1973) FEBS Lett. 34, 280–284.
[2] Kolb, E. and Harris, J. I. (1971) Biochem. J. 124, 76P.
[3] Racker, E. (1950) J. Biol. Chem. 184, 313–319.
[4] Laemmli, U. K. (1970) Nature 227, 680–685.
[5] Percy, M. E. and Buchwald, B. M. (1972) Anal. Biochem. 45, 60–67.
[6] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[7] Jörnvall, H. (1977) Eur. J. Biochem. 72, 425–442.
[8] Bridgen, J., Kolb, E. and Harris, J. I. (1973) FEBS Lett. 33, 1–3.
[8] Biesecker, G., Harris, J. I., Thierry, J. C., Walker, J. E. and Wonacott, A. J. (1977) Nature 266, 328–333.
[10] Spies, J. R. and Chambers, D. C. (1949) Anal. Chem. 21, 1249–1253.