PURIFICATION OF THREONINE DEAMINASE FROM *ESCHERICHIA COLI*

H. GRIMMINGER, I. RAHIMI-LARIDJANI, K. KOERNER and F. LINGENS
Institut für Mikrobiologie und Molekularbiologie der Universität Hohenheim, 7 Stuttgart 70, Germany

Received 3 July 1973

1. Introduction

Biosynthetic threonine deaminase (L-threonine hydrolyase deaminating, EC 4.2.1.16) is the first enzyme in the pathway of isoleucine biosynthesis. Isoleucine and valine are allosteric effectors of this enzyme. The threonine deaminase from *Salmonella typhimurium* has been purified and characterized [1]; it was found to have a mol. wt. of 194 000 and to consist of four identical subunits. The enzyme from *E. coli* has not yet been purified. Affinity chromatography using isoleucine- and valine-Sepharose was shown to be useful for purification of threonine deaminase from *E. coli* [2]; the Sepharose derivatives used in these experiments contained the amino acid attached directly to the matrix and exhibited only weak interactions with threonine deaminase. Cuatrecasas [3] has shown the importance of placing the ligand at some distance from the solid matrix. We tested agarose derivatives obtained after insertion of alkyl 'arms' between agarose matrix and isoleucine. The strength of interaction between Sepharose derivative and threonine deaminase is only slightly increased by an arm which is two carbon atoms long. Derivatives with a 4- or 6-carbon chain adsorb threonine deaminase more strongly.

This paper describes the purification of threonine deaminase from *E. coli* B by affinity chromatography on: 1) valine-Sepharose and 2) isoleucine-*N*-hexamethyleneamine-Sepharose. Preliminary studies indicate that the enzyme has a mol. wt. of about 200 000 and consists of four identical or similar subunits.

2. Materials and methods

Amino acids, pyridoxal phosphate and hexamethylenediamine were obtained from Merck, Sepharose 4B from Pharmacia, Dowex 1X 8 from Carl Roth. Yeast alcohol dehydrogenase, fumarase and catalase were products of Boehringer.

2.1. *Preparation of Sepharose derivatives*

DL-isoleucine-*N*-hexamethyleneamine was synthesized according to Kristiansen et al. [4] and was purified by Dowex-1-formate chromatography. The product was coupled to Sepharose 4B as described by Cuatrecasas [3]. L-valine was coupled directly to Sepharose. Cultivation of the organism: *E. coli* B L 3, leu$^-$ [2] was grown at 37°C in a mineral salt medium containing per liter: $MgSO_4$, 0.2 g; citric acid, 2 g; Na_2HPO_4, 2 g; KH_2PO_4, 5 g; $NH_4H_2PO_4$, 2 g; KOH, 3.4 g; glucose, 2 g and L-leucine, 4.5 mg. Mineral salts were of technical grade. Cells were grown in a 500 ℓ batch fermenter at 37°C with aeration (60 ℓ/min). They were harvested in the late exponential phase and stored at −20°C.

2.2. *Purification*

1.0 M and 0.2 M potassium phosphate buffer pH 8 and 0.01 M potassium phosphate buffer pH 7.4 were used in the purification procedure; these buffers contained 10^{-3} M of each EDTA, 2-mercaptoethanol and isoleucine and 10^{-4} M pyridoxal phosphate.

2.3. *Sonication*

Frozen cell paste (300 g) was suspended in 1 1/2 volumes of cold 1 M phosphate buffer. 100 ml aliquots of the suspension were sonicated for four 1-min periods. The temperature was not allowed to go

Fig. 1. Polyacrylamide gel electrophoresis of purified threonine deaminase. 20 μg protein were subjected to electrophoresis on the standard gel, pH 9.5. Migration was downward.

above 10°C. The cell suspension was centrifuged and the supernatants were pooled.

2.4. *Threonine deaminase activity*

This was determined by measurement of the 2-ketobutyrate formed as described by Umbarger and Brown [5]. Concentration of protein was determined by the biuret method or by measuring the absorbance at 280 nm. Specific activities are expressed as μmole product formed/min per mg protein.

2.5. *Polyacrylamide gel electrophoresis*

The electrophoresis was performed at pH 9.5 using 7.5% gel [6]. Sodium dodecyl sulfate gel electrophoresis was carried out as described by Weber and Osborn [7]. Gels were stained for protein with Amido black 10B.

Table 1
Purification of threonine deaminase.

Fraction	Enzyme (units)	Total protein (mg)	Specific activity	Yield %
Cell extract	10 530	9 200	1.1	100
Valine–Sepharose	7 560	705	10.7	72
Isoleucine-*N*-hexamethyleneamine-Sepharose	5 150	33	155	54
Hydroxylapatite	3 650	16	230	38

2.6. *Sedimentation equilibrium*

Sedimentation equilibrium experiments were performed in the Spinco model E centrifuge equipped with interference optics and an RTIC temperature control unit.

2.7. *Density gradient centrifugation*

Linear sucrose gradients (5 ml, 5–20%) containing 0.02 M potassium phosphate buffer pH 7.4 and 10^{-3} M of each isoleucine, EDTA and mercaptoethanol were overlayered with 0.1 ml of a solution containing threonine deaminase, yeast alcohol dehydrogenase, fumarase and catalase. The tubes were centrifuged in the SW 50 rotor of the Spinco model L 50 centrifuge for 15 hr at 39 000 rpm and 4°C.

3. Results and discussion

3.1. *Purification of threonine deaminase*

Table 1 gives a typical purification procedure. Crude extract was prepared and subjected to valine–Sepharose chromatography [2]. This step results in a 10-fold purification of the enzyme. The active fractions were applied to a column (2.5 × 6 cm) of isoleucine-*N*-hexamethyleneamine-Sepharose. The column was developed with 0.2 M buffer containing 0.5 M KCl and subsequently with 0.01 M buffer. The enzyme was eluted by 0.01 M buffer containing 0.5 M KCl. The active fractions were combined, concentrated and dialyzed against 0.01 M buffer. Specific activity was 150–200 after this step.

The enzyme solution was then applied to a hydroxylapatite column (2.5 × 5 cm) which was equilibrated and developed with 0.01 M buffer; EDTA was omitted

in this step. Threonine deaminase is not retarded by hydroxylapatite. Active fractions were concentrated and the enzyme stored at −20°C. The concentrated enzyme is stable if stored frozen. No loss of activity was detected after several weeks. Total purification was 230-fold, with 35–40% yield of activity.

3.2. *Purity and properties of the enzyme*

Homogenity of the enzyme preparation with respect to molecular weight is concluded from sedimentation equilibrium experiments, since the plots did not show curvature. In SDS-gel electrophoresis only one band was visible (fig. 1). The enzymes from *E. coli* and from *S. typhimurium* showed identical mobility in SDS-gel. When threonine deaminase from *E. coli* was subjected to gel electrophoresis in the standard system and stained for protein, two bands of similar intensity were observed. This is presumably caused by partial dissociation of the enzyme by the Tris buffer of the electrophoresis system.

The purified enzyme exhibits a marked yellow colour. The absorption spectrum shows an absorption maximum at 415 nm, which is characteristic for pyridoxalphosphate-dependent enzymes. The enzyme conserved its kinetic properties during the purification procedure. In density gradient centrifugation the enzyme sedimented as a single peak corresponding to an approximate mol. wt of 190 000. By the meniscus depletion sedimentation equilibrium method a mol. wt. of about 210 000 was obtained. The molecular weight of the polypeptide chains contained in the whole threonine deaminase molecule was determined by gel electrophoresis in the presence of sodium dodecyl sulfate. The molecular weight markers were lysozyme, chymotrypsin, L- and H-chain from bovine globulin and bovine serum albumin. The migration of the threonine deaminase band corresponds to a mol. wt. of about 50 000.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft, the Stiftung Volkswagenwerk, and the Fonds der Chemischen Industrie. The technical assistance of Maria Herrmann is gratefully acknowledged.

References

[1] Burns, R.O. and Zarlengo, M.H. (1968) J. Biol. Chem. 243, 178.
[2] Rahimi-Laridjani, I., Grimminger, H. and Lingens, F. (1973) FEBS Letters 30, 185.
[3] Cuatrecasas, P. (1970) J. Biol. Chem. 245, 3059.
[4] Kristiansen, T., Einarsson, M., Sundberg, L. and Porath, J. (1970) FEBS Letters 7, 294.
[5] Umbarger, H.E. and Brown, B. (1957) J. Bacteriol. 73, 105.
[6] Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404
[7] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406.