Journal of Chromatography, 539 (1991) 507–510
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 897

Use of dye pseudo-affinity chromatography in the purification of homoserine dehydrogenase

M. COSTA-FERREIRA* and J. C. DUARTE
Biotechnology Unit, National Laboratory for Industrial Engineering and Technology (LNETI/DTIQ), Estrada das Palmeiras, 2745 Queluz de Baixo (Portugal)

ABSTRACT

Homoserine dehydrogenase (E.C. 1.1.1.3) from a mutant strain of *Corynebacterium glutamicum* was purified using the pseudo-specific affinity dye Cibacron Blue F3G-A. The triazine dye was coupled either directly or via 1,4-diaminobutane to Sepharose 4B. Both the recovery and purification factor were significantly higher when the dye was coupled to the gel matrix via the spacer. Using this configuration, a recovery of about 62% was obtained with a purification factor of about 95-fold, achieved in a rapid one-step protocol.

INTRODUCTION

Procedures for the purification of homoserine dehydrogenase (E.C. 1.1.1.3) have been reported for various microorganisms [1–4] but these early procedures suffered from various limitations in that they were time consuming and consisted of several tedious steps. More recently, improved purification scheme have been reported for the *E. coli* enzyme using Sepharose substituted with alkyl chains of varying length [5]. Also, a Green A–agarose dye affinity column has been used as the final step in a purification scheme that involved a total of five steps [6].

Our interest in the improvement of *Corynebacterium glutamicum* strains that over-produce amino acids of the aspartate family [7,8] led us to undertake the purification of this key regulatory enzyme. We report here a simple one-step procedure for the purification of homoserine dehydrogenase using the pseudo-specific triazine dye Cibacron Blue F3G-A. A comparison is made between the purification achieved when the dye was immobilized to Sepharose 4B directly and that obtained when immobilized via 1,4-diaminobutane.

EXPERIMENTAL

Reagents

Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden) and Cibacron Blue F3G-A (Reactive Blue), tresyl chloride, 1,4-diaminobutane and Coomassie

0021-9673/91/$03.50

Brilliant Blue from Sigma (St. Louis, MO, U.S.A.). All other reagents were of analytical-reagent grade.

Immobilization of Cibacron Blue F3G-A

In the absence of the spacer arm, coupling was effected by mixing Cibacron Blue (10 μmol/ml gel) with Sepharose 4B suspended in sodium carbonate, using essentially the procedure described previously [9]. The reaction was allowed to proceed for 2 h at 80°C. In the second aternative, the spacer arm, 1,4-diaminobutane, was added in ten-fold excess to tresyl-activated Sepharose 4B [10] and the coupling was run for 1 h at room temperature. After extensive washing, the gel was mixed with Cibacron Blue as described above except that the reaction was allowed to proceed for 1 h at 40°C.

Extraction of homoserine dehydrogenase from Corynebacterium glutamicum

The *C. glutamicum* mutant was grown as described previously [7]. Cells were harvested in the late log phase by centrifugation. The cell pellet was suspended in 100 m*M* potassium phosphate buffer (pH 7.0)–100 m*M* KCl–1 m*M* dithiothreitol (DTT)–1 m*M* EDTA at 4°C. This cell suspension [4% w/v (wet weight)] was disrupted by ultrasonic homogenization (200 W for 4 min). The homogenate was centrifuged (10 000 *g* for 20 min) and the resultant supernatant filtered (0.22-μm filter; Millipore). The cell-free extract (1 ml) was diluted (1:1) with 100 m*M* Tris–HCl (pH 7.0) containing 100 m*M* KCl, 0.5 m*M* DTT, 0.1 m*M* EDTA and 5 m*M* cysteine and applied immediately to a column (30 × 1 cm I.D.) packed with Sepharose 4B–Cibacron Blue previously equilibrated with the same buffer and washed at a flow-rate of 20 ml/h until the absorbance at 280 nm was less than 0.03. Homoserine dehydrogenase was eluted from the column with three column volumes of 2 m*M* nicotinamide–adenine dinucleotide phosphate, oxidized (NADP) in the same buffer system.

Homoserine dehydrogenase activity [11] and protein concentrations [12] were determined using published procedures.

RESULTS AND DISCUSSION

Homoserine dehydrogenase was eluted from the pseudo-affinity column by forming a stable ternary complex [13] with NADP and the competitive inhibitor cysteine [6]. Homoserine dehydrogenase activity was detected in a single essentially symmetrical peak. No homoserine dehydrogenase activity was detected in the large peak containing unbound protein that appeared in the void volume (results not shown).

The results presented in Table I show that the use of Cibacron Blue coupled to Sepharose 4B via the spacer arm gave a significantly higher recovery than when the dye was coupled directly to the gel matrix. In the latter instance the total enzyme activity was about 62%, suggesting incomplete elution. However, neither increasing the NADP concentration 10-fold nor eluting with up to five column volumes resulted in increased recovery. Despite some loss of activity, the yield nevertheless compares favourably with the 24% yield obtained by other workers [6] who used Green A–agarose as the final stage in a multi-step purification scheme.

When Cibacron blue was coupled to the gel via the spacer arm, a 95-fold purification of homoserine dehydrogenase was obtained in a single step. This

TABLE I

PURIFICATION OF HOMOSERINE DEHYDROGENASE FROM *CORYNEBACTERIUM GLUTAMICUM*

Parameter	Crude extract	Cibacron Blue–Sepharose 4B	
		Without spacer	With spacer
Total protein (mg)	8133	75	53
Total activity (U)	244	93	151
Specific activity (U/mg protein)	0.03	1.24	2.85
Recovery (%)	100	38.1	61.9
Purification (-fold)	1	41	95

purification factor is about double than that obtained in the absence of the spacer arm.

In order to exclude the possibility that the higher yield observed may have resulted from higher dye loading on the dye–spacer–Sepharose matrix, the absorbances at 600 nm of the two matrices were determined after gentle heating. As no significant difference was observed between the two matrices, it appears that the total dye load was the same in both instances (Fig. 1).

The higher yield observed in the presence of the spacer suggests that, as has been reported previously for various enzymes such as alcohol dehydrogenase [9], a spacer arm that renders the ligand more flexible is necessary for correct spatial orientation to

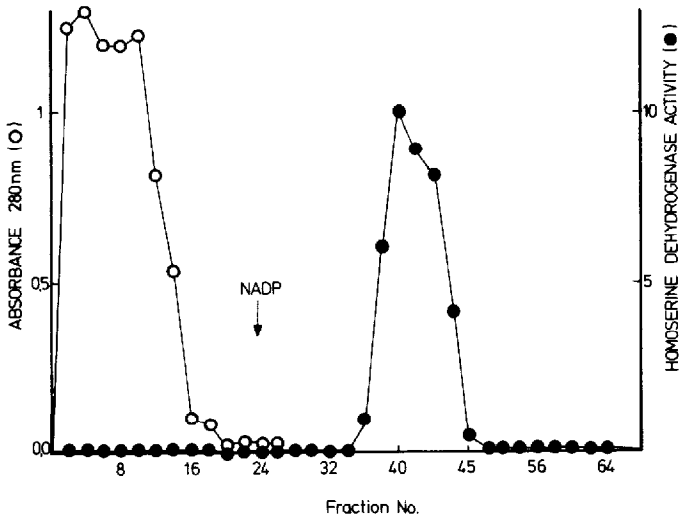

Fig. 1. Dye pseudo-affinity purification of homoserine dehydrogenase. An extract of *C. glutamicum* (244 units of enzyme) was applied to a column of Cibacron Blue F3G-A immobilized onto Sepharose 4B via 1,4-diaminobutane. The homoserine dehydrogenase was eluted with 2 m*M* NADP (as indicated by arrow) in 100 m*M* Tris–HCl (pH 7.0) containing 100 m*M* KCl, 0.5 m*M* DTT, 0.1 m*M* EDTA and 5 m*M* cysteine. Fractions (2 ml) were collected and enzyme activity was assayed as described under Experimental.

homoserine dehydrogenase, whereas in the absence of the spacer steric factors hinder access to the ligand.

Work is in progress to optimize the purification by avoiding problems associated with the use of unpurified dyes [14], studying the effect of using longer spacer arms such as diaminohexane and studying the many other factors that affect purification efficacy.

ACKNOWLEDGEMENTS

We thank Dr. Torbjörn Ling of the Department of Biotechnology, University of Lund, Sweden, for helpful advice on the use of triazine dyes.

REFERENCES

1 N. Guiso and P. Truffa-Bachi, *Eur. J. Biochem.*, 42 (1974) 401.
2 M. Shailaja and M. R. Raghavendra Rao, *Mol. Cell. Biochem.*, 12 (1976) 15.
3 P. Datta, *J. Biol. Chem.*, 245 (1970) 5779.
4 M. Jenkins and V. Woodward, *Biochim. Biophys. Acta*, 212 (1970) 21.
5 L. Broussard, W. Harms and W. Shive, *Anal. Biochem.*, 72 (1976) 16.
6 W. Karsten, J. Hunsley and R. Viola, *Anal. Biochem.*, 147 (1985) 336.
7 M. Costa-Ferreira and J. C. Duarte, *Appl. Biochem. Biotechnol.*, (1991) in press.
8 M. Costa-Ferreira and J. C. Duarte, *Port. Pat. Appl.*, No. 91 207, 1989.
9 B. Mattiasson and T. G. I. Ling, *J. Chromatogr.*, 376 (1986) 235.
10 K. Nilsson and K. Mosbach, *Methods Enzymol.*, 104 (1984) 56.
11 P. Datta and H. Guest, *Methods Enzymol.*, 2 (1970) 703.
12 M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
13 G. Svanas and H. Weiner, *Anal. Biochem.*, 124 (1982) 314.
14 B. Weber, K. Willeford, J. G. Moe and D. Piszkiewicz, *Biochem. Biophys. Res. Commun.*, 86 (1979) 252.