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Note

Purification of aldehyde reductase 1 from pig liver

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Aldehyde reductases (ALR 1, E.C. 1.1.1.2 and ALR 2, E.C. 1.1.1.21) are monomeric NADPH-dependent enzymes which play important physiological roles[1,2]. Several purification procedures for the preparation of homogeneous aldehyde reductases from mammalian tissues have been described[3–7]; ALR 1 from pig liver and kidney has been purified by Branlant and Biellmann[3] and by Morpeth and Dickinson[4], respectively. These purification procedures involved a complex series of chromatographic separations. The present paper describes a simplified isolation procedure for ALR 1 which uses two high-performance chromatographic steps.

EXPERIMENTAL

Materials

Pig livers were obtained from the slaughter house and were stored at −60°C. Commercial NADPH (Reanal, Hungary) containing about 85% of this substance was purified as described[8]. Pyrazole (Aldrich, Milwaukee, MI, U.S.A.), *p*-nitrobenzaldehyde and mercaptoethanol (Fluka, Buchs, Switzerland) were used without further purification. The other chemicals were of analytical purity.

Enzyme preparation

The initial procedures were carried out at 4°C, the final high-performance separations proceeded at room temperature (the eluted fractions being kept in an ice-bath). All buffers contained 0.1 m*M* mercaptoethanol and 0.05 m*M* EDTA.

The initial purification steps (*i.e.* homogenization and ammonium sulphate fractionation) were carried out similarly, as described[3,4]. The sample obtained by ammonium sulphate fractionation was dialysed against 0.05 *M* sodium phosphate buffer (pH 7) and applied to a column (250 × 50 mm I.D.) packed with Matrex Gel Blue A (Amicon, Oosterhout, The Netherlands), with a flow-rate of *ca.* 0.1 ml/min. The column was washed with the same buffer until the absorbance at 280 nm decreased below 0.1. Thereafter, a gradient of sodium chloride (0–1.2 *M*) in the above buffer was used. The active fractions were pooled and the protein was precipitated with ammonium sulphate. The precipitate was dissolved in 15 m*M* Tris–HCl buffer (pH 8) and applied to a column (1000 × 50 mm I.D.) containing Sephadex G-75 (Pharmacia, Uppsala, Sweden), at a flow-rate of *ca.* 0.15 ml/min. The same buffer was used for the elution; the active fractions were collected and concentrated by means of an Amicon ultrafiltration cell (with a YM-10 membrane).

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TABLE I

PURIFICATION OF ALDEHYDE REDUCTASE 1 FROM PIG LIVER

Starting material was 0.2 kg of liver; the details of the procedure are described in the text; values are averages from three preparations.

Fraction	*Protein (mg)*	*Total activity (U)*	*Specific activity (U/mg)*	*Purification*	*Recovery (%)*
Crude supernatant	68 000	408	0.006	(1)	(100)
Ammonium sulphate fractionation (30–75%)	15 000	285	0.019	3.3	70
Matrex Gel Blue A (+ concentration)	970	223	0.24	40	57
Sephadex G-75 (+ concentration)	160	195	1.22	200	48
Mono Q column (+ concentration)	15	165	11.3	1900	40
UltroPac TSK 3000 SW column	8	144	18.8	3100	35

The next step was the ionex chromatography on a Mono Q HR 5/5 column (Pharmacia) attached to two P-500 pumps and a GP-250 gradient programmer (Pharmacia). As starting and terminating buffers, 15 m*M* Tris–hydrochloric acid (pH 8) and the same buffer with 1 *M* sodium chloride were used (flow-rate = 1.5 ml/min). The separations were evaluated by a UV-1 monitor (λ = 280 nm) and a FRAC-100 collector (Pharmacia). The active fractions were pooled and concentrated as given above. The sample was applied to an UltroPac TSK 3000SW column (LKB, Bromma, Sweden) attached to the same chromatographic system, and 0.2 *M* sodium phosphate buffer (pH 6.8) was used as the mobile phase (flow-rate = 1.1 ml/min).

Enzyme analysis

The enzymatic activity of ALR was determined by measuring the oxidation rate of NADPH (0.1 m*M*) using *p*-nitrobenzaldehyde (2 m*M*) in 0.1 *M* sodium-phosphate buffer (pH 7) at 25°C. The assay mixtures contained 0.5 m*M* pyrazole to inhibit the alcohol dehydrogenase activity[9]. The other dehydrogenase activities were assayed as described[10]. Protein concentrations were estimated spectrophotometrically[3] in a Cary 118 apparatus (Varian, Palo Alto, CA, U.S.A.). The purified enzyme was analysed by chromatofocusing and polyacrylamide gel electrophoresis[11].

RESULTS AND DISCUSSION

The results of the initial purification steps (Table I) were comparable with those reported (*cf.* refs. 3 and 4). Chromatography on Matrex Gel Blue A (containing Cibacron Blue 3GA as the affinant[12]) was used as the first rather selective separation step in the described isolation procedure. Aldehyde reductase showed a high affinity to this bio-specific resin; most of the other proteins binding to this material (*e.g.* malate dehydrogenase and essential amounts of lactate dehydrogenase and alcohol dehydrogenase) were eluted at lower sodium chloride concentrations. This affinity step proved to be very convenient since the increase in the specific activity of ALR was greater than ten-fold and the activity decrease was small (Table I). Chromatography on a Sephadex G-75 column represented the following step in the proposed purification procedure (*cf.* ref. 4). Our results indicated that the liver ALR was eluted at higher elution volumes than the main protein fractions of the partially purified sample (containing especially the remaining major dehydrogenase activities). The purification was *ca.* five-fold, the recovery being higher than 80% (Table I). The values of the specific activity showed, however, that the preparation contained more than 90% of the contaminating proteins (*cf.* Table I).

Chromatography on a Mono Q column (Fig. 1) provided an excellent method for the separation of most of the contaminating proteins with both more acidic and more alkaline properties in comparison with ALR. The high efficacy of this chromatography was documented by the fact that the increase in specific activity was nearly ten-fold (Table I). Moreover, this step made it possible to separate ALR 1 from ALR 2. ALR 1 appeared as a high protein peak at low values of sodium chloride concentration, whereas ALR 2 (*ca.* 5% of the total ALR activity) was eluted as a less pure zone at slightly higher sodium chloride concentrations (Fig. 1). Owing to the negligible ALR 2 activity, we did not try to purify this enzyme to homogeneity. The final step in the purification of ALR 1 (*i.e.* chromatography on a TSK 3000SW

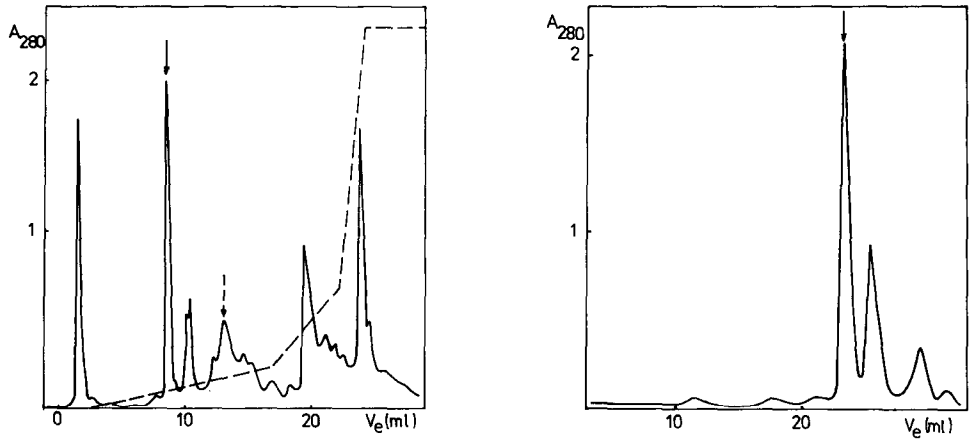

Fig. 1. Chromatography of partially purified aldehyde reductase on a Mono Q column. Buffer A, 15 m*M* Tris–HCl (pH 8.0); buffer B, the same with 1 *M* sodium chloride. V_e, elution volume; ———, absorbance at 280 nm (A_{280}); ---------, sodium chloride gradient; full and broken arrows correspond to high (ALR 1) and low (ALR 2) enzyme activities, respectively. Approximately 5 mg of protein were applied to the column; in semi-preparative runs *ca.* six-fold amounts were used.

Fig. 2. Chromatography of aldehyde reductase 1 on a UltroPac 3000SW column. Mobile phase, 0.2 *M* sodium phosphate buffer (pH 6.8); the symbols are as in Fig. 1. Approximately 3 mg of protein were injected; in semi-preparative runs *ca.* two-fold amounts were used.

column) revealed that the sample eluted from the Mono Q column contained negligible traces of proteins with a molecular weight range exceeding that of ALR 1 (*i.e.* the molecular weight range of all NAD- and NADP-dependent dehydrogenases[1,3]) (*cf.* Fig. 2). On the other hand, the content of smaller proteins and peptides in this sample was still significant. However, these impurities were completely removed in the final purification step (Fig. 2).

Aldehyde reductase 1 from pig liver prepared by the proposed method proved to be a single protein. Only one protein peak was observed in repeated runs on Mono Q and TSK 3000SW columns, by chromatofocusing and by SDS-polyacrylamide gel electrophoresis. The values of the molecular weight of the purified protein (35 000 ± 3000 daltons by gel permeation chromatography and 37 000 ± 2000 daltons by sodium dodecyl sulphate–polyacrylamide gel electrophoresis) were comparable with those published for ALR 1 from pig liver and pig kidney[3,4]. The enzymatic properties of our preparation (Michaelis constant for *p*-nitrobenzaldehyde = 0.25 m*M* at pH 7, strong inhibition with barbiturates, negligible inhibition with pyrazole and no activity when NADPH was replaced with NADH) were identical with those reported (*cf.* refs. 3 and 4).

The results presented here document the advantages of high-performance chromatographic methods in final phases of protein purifications. The proposed method is essentially simpler and shorter than the methods described[3–7]. The proposed final high-performance chromatographic separations yielded a homogeneous enzyme (the purification being about fifteen-fold in comparison with the specific activity of the sample obtained in the last large-scale step, *i.e.* in the chromatography on Sephadex G-75 (*cf.* Table I). The results of the semi-preparative separations presented here

were obtained with relatively small high-performance columns (repeated runs yielded *ca.* 10 mg of protein in *ca.* 3–4 h). The use of larger commercially available high-resolution columns (such as Mono Q HR 16/10 and UltroPac 3000SWG) is preferable for the preparation of this enzyme at larger quantities. The enzyme prepared by the described method is suitable for the most accurate kinetic or equilibrium binding experiments and for an exact determination of its physical and chemical properties.

REFERENCES

1 N. Bachur, *Science*, 193 (1976) 595.
2 T. G. Flynn, *Bull. Can. Biochem. Soc.*, 20 (1983) 38.
3 G. Branlant and J. F. Biellmann, *Eur. J. Biochem.*, 105 (1980) 611.
4 F. F. Morpeth and F. M. Dickinson, *Biochem. J.*, 191 (1981) 619.
5 G. Branlant, *Eur. J. Biochem.*, 129 (1982) 99.
6 N. Takahashi, T. Saito and K. Tomita, *Biochim. Biophys. Acta*, 748 (1983) 444.
7 C. H. Ryle, T. Dowling and K. F. Tipton, *Biochim. Biophys. Acta*, 791 (1984) 155.
8 G. A. Orr and J. S. Blanchard, *Anal. Biochem.*, 142 (1984) 232.
9 C. I. Brändén, H. Jörnvall, H. Eklund and B. Furugren, in P. D. Boyer (Editor), *The Enzymes*, Vol. 11, Academic Press, New York, 1975, 3rd ed., p. 103.
10 *Biochemica Information I*, Boehringer, Mannheim, 1973.
11 I. Matysková, J. Kovář and P. Racek, *Biochim. Biophys. Acta*, 839 (1985) 300.
12 *Matrex Gels*, Amicon Corporation, Oosterhout, The Netherlands, 1980.
13 M. G. Rossman, A. Liljas, C. I. Brändén and L. J. Banaszak, in P. D. Boyer (Editor), *The Enzymes*, Vol. 11, Academic Press, New York, 1975, 3rd ed., p. 6.