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Note

Rapid chromatographic purification of yeast alcohol dehydrogenase

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Various procedures for the preparation of alcohol dehydrogenase from yeast (YADH, E.C. 1.1.1.1) have been described[1–5]. Those using repeated ammonium sulphate or ethanol precipitations[1,2] of the yeast cytolysate are relatively simple and suitable for large scale preparations. DEAE-cellulose chromatography included in some purification procedures (*cf.*, ref. 5) improves the separation of the main YADH isoenzyme from the minor isoenzymes[6]; the purification process is inevitably prolonged. The aim of this paper is to propose a method involving two rapid and efficient chromatographic steps together with the initial step of one of the widely used procedures[1]. The described method is suitable for a quick semi-preparative purification of the main YADH isoenzyme.

EXPERIMENTAL

Chemicals

NAD was obtained from Boehringer (Mannheim, F.R.G.), commercial yeast alcohol dehydrogenase from Sigma (St. Louis, MO, U.S.A.). Polybuffers 96 and 74 were obtained from Pharmacia (Uppsala, Sweden). The other chemicals (mostly from Lachema, Brno, Czechoslovakia) were of analytical purity.

Enzyme preparation

YADH was isolated from baker's yeast, using the procedure described by Butler and Jones[1]. The yeast cells were broken by toluene plasmolysis and the extract was fractionated by successive additions of ammonium sulphate. The protein fraction obtained at 60% saturation was crystallized upon addition of ammonium sulphate to saturation. These "first crystals" (according to ref. 1) could be stored for several months at 4°C without appreciable losts of enzymatic activity.

The next step was chromatofocusing on a Mono P HR 5/20 column (200 × 5 mm, Pharmacia) attached to two P-500 pumps (Pharmacia). As starting and terminating buffers, 25 m*M* triethanolamine pH 8.3, and a mixture of Polybuffers 96 (3%) and 74 (7%) adjusted to pH 5.0 with iminodiacetic acid were used. The protein sample obtained from the first crystallization was dissolved in and dialysed against the starting buffer prior to chromatofocusing. The separations were evaluated by means of a UV-1 monitor (λ = 280 nm), a flow-through pH electrode and a FRAC-100 collector (Pharmacia). The fractions containing the main isoenzyme of

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YADH were pooled, concentrated with an Amicon (Oosterhout, The Netherlands) ultrafiltration cell and applied to an UltroPac TSK 3000SW (or TSK 3000SWG) column (LKB, Bromma, Sweden) attached to the same chromatographic system described above. A 0.2 *M* sodium phosphate buffer pH 7.0 containing 10 μM ethylene diaminetetraacetate (EDTA) and 10 μM mercaptoethanol was used as the mobile phase. The results were evaluated as mentioned above. The fractions containing the highest YADH activity were pooled, concentrated with an ultrafiltration cell, precipitated by ammonium sulphate or used directly for further experiments. The chromatographic separations proceeded at room temperature, the fractions eluted from the columns being stored at 4°C.

The purity of the enzyme was tested both by determination of the specific activity (enzymatic activity and protein concentration measured according to ref. 2 using a Cary 118 spectrophotometer, Varian, Palo Alto, CA, U.S.A.) and by chromatographic analysis using the conditions recommended in ref. 7.

RESULTS AND DISCUSSION

The initial steps in the purification procedure (*i.e.*, extraction, salting-out and first crystallization) yielded comparable results to those described previously[1], *cf.*, Table I. In principle, it is also possible to prepare the crude enzyme sample by other procedures, *e.g.*, by precipitation with ethanol (which has been claimed to yield enzyme of specific activity exceeding 40 units per mg protein[2]). Larger amounts of the partially purified enzyme can be stored in a refrigerator for several months and used as the source of YADH for the subsequent semi-preparative chromatographic procedure.

TABLE I

PREPARATION OF ALCOHOL DEHYDROGENASE FROM BAKER'S YEAST

The data are averages calculated from three experiments with *ca.* 0.5 kg yeast.

Fraction	*Protein (mg)*	*Specific activity (units/mg)*	*Yield in each step (%)*
Extract	25000	2.2	–
Precipitate from $(NH_4)_2SO_4$, dissolved	6450	6.8	80
First crystals	800 (150*)	27	49
After chromatofocusing	24.5	160	90
After TSK 3000SWG chromatography	9	395	88

* Only 150 mg of the protein were taken for the following chromatographic separations.

The results of the efficient chromatofocusing technique, proposed as the next purification step, are shown in Fig. 1A. The elution profile indicates that the separation of YADH from most of the contaminating proteins was very good and that the main enzyme activity was eluted in a high peak at about pH 5.9 (which is in a good accord with the published value[8] of the isoelectric point of the main YADH isoenzyme, 5.8). The minor YADH activities (less than 20% of that of the main isoenzyme) were detected in two other peaks (eluted at both higher and lower pH

values in comparison with the main isoenzyme, *cf.*, Fig. 1A). The increase in the specific activity of YADH achieved in this step was 5–7-fold, the recovery of the main isoenzyme being about 90%. The experiment was usually repeated four to six times to yield about 3000–4000 units of the main isoenzyme of YADH (corresponding to more than 20 mg protein).

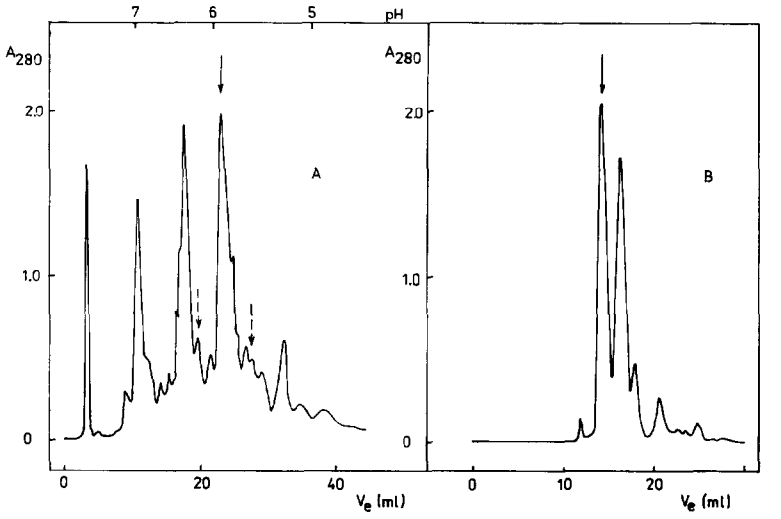

Fig. 1. Final steps in the purification of yeast alcohol dehydrogenase. The separation conditions are described in Experimental. The peaks containing YADH activities are indicated by arrows, full and broken arrows corresponding to high and low activities, respectively. V_e = Elution volume. A, Chromatofocusing on a Mono P HR 5/20 column (about 25 mg of total protein, flow-rate 1.4 ml/min); B, gel permeation chromatography on a UltroPac TSK 3000SW column (about 5 mg of total protein, flow-rate 1.2 ml/min).

As the final step of YADH purification, chromatography on TSK 3000SW or TSK 3000SWG columns was carried out. This separation method is a very efficient and rapid variant of gel permeation chromatography; it separates the enzyme from the remaining contaminating proteins, which are still present in the sample after chromatofocusing (see Fig. 1A), and also removes the inactive (denatured) fragments of the enzyme active tetramer (M_r = 140000, *cf.*, ref. 6) and the components of the chromatofocusing medium. The mobile phase used in this separation contained small amounts of EDTA and mercaptoethanol to prevent denaturation of the enzyme. When these compounds were not present in the buffer, slightly lower yields of the enzyme activity were attained. A typical separation on a semi-preparative column (TSK 3000SW, 600 × 7.5 mm) is shown in Fig. 1B. The enzyme was purified 2.5–3-fold, the yield of the activity being about 90%. The resolution achieved with a larger column (TSK 3000SWG, 600 × 21.5 mm) was slightly worse, the increase in enzyme activity being about 2.5-fold). However, it was possible to purify the whole amount of the protein with YADH activity in six consecutive chromatofocusing experiments (more than 20 mg protein) in only one step.

The results of the proposed purification procedure are summarized in Table I. The efficacy and duration of the described method are comparable with those of

the best known methods for YADH preparation[1–5]. The whole chromatographic separation (including six chromatofocusing steps, continuous ultrafiltration of the eluted fractions with YADH activity and one final chromatography on a TSK 3000SWG column) takes about 4 h and yields about 10 mg of the pure main isoenzyme of YADH (Table I). The specific activity of the final product is higher than that obtained with commercial preparations and is comparable to that given for the enzyme purified by ethanol precipitation and repeated crystallizations from ammonium sulphate solutions[2]. Moreover, the proposed method yields the main YADH isoenzyme as a single protein. This was shown by chromatographic analysis of the purified enzyme using the described conditions[7]. The enzyme sample was eluted as one protein peak which contained the total enzyme activity, in contrast with the commercial preparation which contained traces of impurities (not shown). The described method proved suitable for the preparation of smaller amounts of YADH which could be used directly for measurements requiring a highly purified protein, *e.g.*, for kinetic, spectrophotometric or spectrofluorometric experiments.

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