PURIFICATION AND CHARACTERIZATION OF ALCOHOL DEHYDROGENASE FROM PIG LIVER

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Alcohol dehydrogenase from pig liver was purified from a tissue homogenate under conditions of enzyme protection in a complex with NADH* and isobutyramide and further by chromatography on ion-exchange celluloses and Sephadex G-100. pH 8 and an addition of dithiotreitol was found most effective for stabilization of the enzyme. Electrophoretic behavior revealed the existence of at least 4 isoenzymes with variable relative amounts of 3 minor components. Using a titration of the enzyme by NADH in the presence of isobutyramide the concentration of active sites and turnover number of the enzyme were determined. The basic kinetic characteristics show that the pig enzyme is less active than that of the horse origin, whereas its affinity to coenzymes and substrates is comparable. Pig alcohol dehydrogenase can be inhibited by inhibitors active against other animal alcohol dehydrogenases.

Alcohol dehydrogenases of animal origin have been extensively investigated[1,2]. The methods for their isolation were worked out, and the kinetics of enzymic action and the effect of inhibitors were analyzed in detail. Amino acid sequences of alcohol dehydrogenases and tertiary structures based on X ray analysis data were determined in several cases[1]. The best known enzyme is the alcohol dehydrogenase from horse liver followed by the enzymes isolated from rat and human liver. On the other hand the data concerning alcohol dehydrogenase from pig liver are less numerous[2,3]. This enzyme has not been purified and its kinetic behavior has not been tested. It was the aim of this work to work out a method for the purification of ADH from pig liver and to characterize the purified enzyme.

EXPERIMENTAL

Isolation and purification of ADH *from pig liver.* Fresh or frozen pig liver (usually 400 g) was used. To this material 100 ml of distilled water was added and homogenized for 2 minutes. After addition of 1 ml of 0·5M-$ZnSO_4$ and adjustment to pH 8 by ammonium hydroxide the homogenate was stirred for 60 minutes at laboratory temperature. Measured activity (53 nkat per 1 g of tissue) was consistent with the data in the literature[3]. The enzyme in homogenate was converted into a ternary complex with NADH and isobutyramide (designated further as ERI)

* Abbreviations used: NADH, nicotinamide adenine dinucleotide; ADH, alcohol dehydrogenase; ERI, ternary complex enzyme–NADH–isobutyramide.

by addition of isobutyramide to a final concentration 0·05M and, immediately before heating the mixture, by addition of NADH to a concentration 2 to 3 times higher than the assumed enzyme concentration. Protein fractionation by heat denaturation was carried out by gradually increasing the temperature of the homogenate to 40°C followed by an increase to 70°C during 1—2 minutes and by keeping the solution for further 2 minutes at that temperature under stirring. After rapid cooling the denatured proteins were pelleted by centrifugation (60 min, 2000*g*) and the supernatant was concentrated by evaporation under reduced pressure (1 to 3 kPa) at 30–40°C to one seventh of its original volume. The enzyme was salted out by 100% saturation with ammonium sulphate and pH was adjusted to 8 by addition of ammonium hydroxide. The pelleted proteins were suspended in a small amount of phosphate buffer pH 8 (*I* 0·1) and were stored for further use. 50 ml of this fraction were dialysed for 3 hours against water and overnight against twice 5 l of sodium phosphate buffer, pH 8 (*I* 0·02). After adjusting the pH to 6 and after the conversion of the enzyme into ternary ERI complex (volume 250 ml) the solution was applied to a CM cellulose column (39 × 160 mm) equilibrated with sodium phosphate buffer, pH 6 (*I* 0·05). The flow rate was 2 ml per minute and the eluate after adjustment to pH 8 was concentrated using an Amicon model 202 apparatus with a Diaflo XM 50 membrane to one tenth of its original volume. Decomposition of the ERI ternary complex and adjustment of the solution ionic strength and pH were carried out by chromatography on a 39 × 200 mm Sephadex G—25 column equilibrated with 0·05M-Tris-HCl buffer, pH 8·4, using a flow rate of 5 ml per minute. The resulting solution (65 ml) was applied on a 39 × 150 mm DEAE Sephadex A—50 column equilibrated with 0·05M-Tris-HCl buffer, pH 8·4, using a flow rate of 4 ml per minute. After adjustment to pH 8 the purest fraction (100–120 ml) was concentrated to 17 ml using an Amicon model 202 apparatus with a Diaflo XM 50 membrane. 1 ml of this solution was applied on a 10 × 550 mm Sephadex G—100 column (equilibrated with 0·1M-Tris-HCl buffer, pH 8) using a flow rate of 0·1 ml per minute and the enzymic activity was found in the second peak (Fig. 1). All procedures were carried out at 5°C.

Electrophoretic separation. Best results were obtained by agarose gel electrophoresis at pH 9·5, 0·05M-Tris-HCl buffer, pH 9·5, was used for separation and the electrode compartments were filled with the same buffer in 0·1M concentration. In all cases 3 ml of 1% agarose (Koch-Light) in 0·05M-Tris-HCl buffer, pH 9·5, were put on a glass plate (77 × 27 mm). At the starting position an incision was made and filter-paper strips saturated with the sample were inserted in. The separation proceeded for 30—40 minutes at a constant voltage 500—600 V and with 2·5 mA at one layer. The agarose gel electrophoresis at pH 7 was carried out accordingly. Sodium phosphate buffer, pH 7 (*I* 0·1) was used in the electrode compartments. The electrophoresis of every sample was performed in two layers in parallel. One layer was used for the activity assay, in the other one protein was determined. The detection of activity was carried out for 60 minutes at 37°C using a mixture in glycine buffer, pH 10, containing $5 \cdot 10^{-3}$M nitrobluetetrazolium chloride, $1{\cdot}1 \cdot 10^{-4}$M phenazinemethosulphate, $2{\cdot}5 \cdot 10^{-2}$M ethanol and $1{\cdot}25 \cdot 10^{-3}$M-NAD^+. Proteins were detected by 1% amidoblue 10B in 7% acetic acid for 40 minutes and the dye was removed from the layer by 7% acetic acid. All layers were fixed by 2% acetic acid and dried. Before electrophoresis the homogenate was centrifuged for 45 minutes at 20000*g* in a centrifuge Christ Omega II. The separation of low molecular weight substances was carried out on a Sephadex G—25 column equilibrated with 0·1M-Tris-HCl buffer, pH 9·5, and the solution was concentrated in a collodium bag SM 13200 (Sartorius) under vacuum using a water jet pump.

Measurement of enzyme activity. The activity of ADH with ethanol was measured at 340 nm and 25°C in a 1 cm cell containing 1 mM-NAD^+, 8·6 mM ethanol in 0·07M-NaOH–glycine buffer, pH 10·5, in a total volume of 3 ml, in a Varian Cary 118 spectrophotometer. The activity with ethanol at pH 7 was measured accordingly in a mixture containing 0·8 mM-NAD^+ and 15 mM

ethanol in a sodium phosphate buffer, pH 7 (I 0·1). The enzyme activity was determined according to Dalziel[4] (the method was modified using the known turnover number of pig ADH) and is expressed as enzyme concentration in mg/ml (or μM), or it was calculated from the initial reaction rate and is expressed in katals. Kinetic studies were carried out using a Varian Cary 118 spectrophotometer, fluorometric measurements were performed in an Aminco-Bowman spectrofluorometer.

Titration of enzyme binding sites with NADH in the presence of isobutyramide was done according to Yonetani and Theorell[5]. Molecular weight determination of ADH was performed using thin-layer gel chromatography with Sephadex G—100, superfine grade, in 0·1M-Tris-HCl buffer, pH 8·4 (ref.[6]). Molecular weight of ADH from pig liver was compared with that of horse liver ADH and with other standards as ferritin, bovine serum albumin, aldolase, ovalbumin and chymotrypsinogen. Protein content was determined both by the method of Lowry and coworkers[8] and from the absorbance at 280 nm (ref.[7]).

Materials. NAD^+ was a product of Sevac, NADH was from Reanal or Merck and isobutyramide was synthesized according to Kent and Elvain[9]. Pyrazole was a product of Aldrich, reduced glutathione was from Merck, dithiotreitol from Sigma, and nitrobluctetrazolium chloride, phenazinemethosulphate and phenantroline were purchased from Lachema. Berberine sulphate was from E. Gurr, ethylberberine chloride and chlorprothixene were characterized earlier[10]. CM-cellulose CM—32 was from Whatman, Sephadex G—25, G—100, G—100 superfine and DEAE A-50 were obtained from Pharmacia. Ethanol, propanol, n-butanol, cyclohexanol and acetaldehyde were redistilled, other reagents were of analytical grade.

RESULTS AND DISCUSSION

The method of alcohol dehydrogenase isolation by fractionation using heat denaturation under conditions of enzyme protection in a heat stable ternary complex enzyme–NADH–isobutyramide (ERI) introduced for the purification of ADH from horse liver, was successfully used for the isolation of ADH from pig liver with a yield of about 90%. For further purification of the enzyme from pig liver salting out by ammonium sulphate cannot be applied because of simultaneous increase of the content of all proteins together with the enzyme in the precipitate on increasing the degree of saturation. However, ammonium sulphate precipitation turned out to be useful for a long-term storage of the enzyme.

Chromatographic techniques were used for further purification of ADH from pig liver. In contrast to the ADH from horse liver, ADH from pig liver in the free state is not retained on a CM-cellulose column equilibrated with sodium phosphate buffer, pH 6 (I 0·05). However, a considerable portion of accompanying basic proteins remains on the column and this type of chromatography was used as a purification step. During this purification step, ADH was present as a more acidic ternary complex ERI. Most acidic proteins were retained on a DEAE Sephadex A-50 column equilibrated with 0·05M-Tris-HCl buffer, pH 8·4 and the enzyme purified more than 100 times was obtained.

Chromatography on Sephadex G—100 (Fig. 1) was used as a supplementary purification step in order to get rid of an accompanying protein similar to the enzyme,

which could not be separated by agarose gel electrophoresis at pH 9 and 7. The peak of the non-enzymic protein was less pronounced in those cases of isolation procedure, in which a better enzyme purification was achieved by DEAE Sephadex A–50 column chromatography (Fig. 1). In any case, however, the purest fraction from Sephadex G–100 column contained the enzyme purified more than 200 times, of about 70% purity. The isolation procedure is summarized in Table I.

For the calculation of enzyme purity the known turnover number of the enzyme and its molecular weight were used. A comparison of the spectrophotometric method of protein content determination with the method of Lowry and coworkers[8] shows that the absorption coefficient of ADH from pig liver at 280 nm is relatively low, not higher than 0·5 ml mg^{-1} cm^{-1}. This

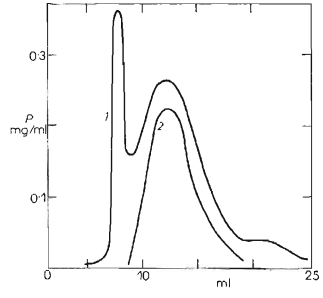

FIG. 1

Chromatography of Pig Alcohol Dehydrogenase on Sephadex G–100

1 Total protein concentration (determined from A_{280} and by the Lowry method), *2* concentration of the enzyme protein (determined by enzyme activity measurement). ml, elution volume.

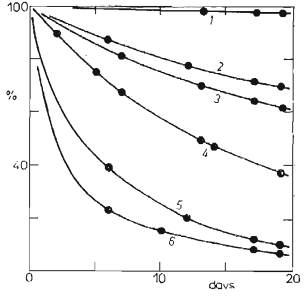

FIG. 2

Stability of Pig Alcohol Dehydrogenase at Various Purification Stages

%, Relative activity; days, time of incubation. *1* the precipitate after salting out with ammonium sulphate (pH 8, —15°C), *2* the precipitate after salting out with ammonium sulphate dissolved in sodium phosphate buffer, pH 8 (*I* 0·1, —15°C), *3* liver tissue homogenate (pH 7·5, —15°C), *4* after chromatography on DEAE Sephadex A—50, solution concentrated (0·05M-Tris-HCl buffer, pH 8, 0°C), *5* ternary complex with NADH and isobutyramide (pH 8, —15°C) and *6* dialysed diluted solution in phosphate buffer, pH 8 (*I* 0·05, 0°C).

value is similar to that of the respective absorption coefficient of ADH from horse liver and shows that both enzymes contain few tryptophane residues[1].

The stability of the enzyme in all forms corresponding to the individual purification stages is highest at pH 8 (Fig. 2). The ternary complex ERI, which is relatively stable at elevated temperatures, decomposes rather rapidly at 0°C during a long-term storage. In contrast to ADH from horse liver, the stability of pig liver enzyme in the ternary complex is even lower at —15°C (Fig. 2). The precipitate salted out by 100% saturation with ammonium sulphate, which retains its activity unchanged for several months at —15°C (Fig. 2), was found to be a suitable form for storage of the ADH from pig liver. The stability of ADH in solution can be increased by addition of compounds protecting SH groups, this protection being most pronounced in the presence of dithiotreitol in contrast to glutathione, where the effect was negligible (Table II).

TABLE I

Purification of Pig Liver Alcohol Dehydrogenase

Purification stage	Purity %	Degree of purification	Yield %
Liver homogenate	0·3	1	100
Heat denaturation	2·3	7	90
Dialysed precipitate after salting out by 100% saturation with $(NH_4)_2SO_4$	2·5	8	85
Chromatography on CM-cellulose	5·0	16	67
Chromatography on DEAE Sephadex A—50	30—40	100—130	45
Chromatography on Sephadex G—100	50—70	170—230	30

TABLE II

The Effect of Glutathione and Dithiotreitol on the Stability of Pig Liver Alcohol Dehydrogenase

The enzyme (10 μM) was kept at 4°C in sodium phosphate buffer, pH 8, in the presence of 15 mM dithiotreitol and 7 mM glutathione. Enzyme activity was measured in 0·07M-NaOH–glycine buffer, pH 10·5, at 25°C. The results are averages of three measurements.

Incubation days	Relative activity, %		
	control	glutathione	dithiotreitol
0	100	100	100
3	66	70	103
6	36	42	106
9	25	30	89
12	20	25	95
15	18	19	90

By electrophoretic separation in agarose gel at pH 9·5 of a preparation from liver tissue 4 ADH isoenzymes were detected, 3 of which move to the anode and are present in lower amounts than the main isoenzyme moving to the cathode. The detection of four isoenzymes in the case of pig liver ADH makes a correction in the existing literary data[3]. The relative amounts of anodic isoenzymes were found to vary with different individuals. In the course of the isolation procedure the activities of all anodic isoenzymes gradually disappear. During electrophoresis in agarose gel at pH 9·5 (as well as at pH 7) the purified enzyme revealed only one zone of the most abundant isoenzyme moving to the cathode. The accompanying proteins could not be separated from it. It was established that none of the isoenzymes is of mitochondrial origin.

Using the method of thin-layer gel chromatography the approximate molecular weight of ADH from pig liver was found to be 78000 to 82000 daltons, as in the case of the horse liver enzyme. This suggests that similarly to other animal alcohol dehydrogenases the pig liver ADH forms a dimer of molecular weight about 80000 daltons consisting of two subunits with molecular weight about 40000 daltons.

Fluorometry was used following the interaction of the enzyme (70% purity) with NADH and isobutyramide, which in the case of other alcohol dehydrogenases acts as an inhibitor competitive with respect to aldehyde. In Fig. 3 fluorescence emission spectra of a binary complex ADH–NADH and of a ternary complex ERI (with isobutyramide added) are shown and compared with fluorescence emission spectra of free NADH. Similarly as in the case of ADH from horse liver[5], an increase of fluorescence intensity and a blue shift of the emission maximum take place with the pig enzyme. These effects might suggest the hydrophobic nature of the enzyme active center.

Taking advantage of the stable complex ADH–NADH–isobutyramide the concentration of enzyme binding sites for NADH (*i.e.* active centers or active enzyme subunits) can be evaluated by so called isobutyramide titration (Fig. 4), as described for the horse liver ADH (ref.[5]). The determination of enzyme active site concentration makes it possible to calculate exact concentration of the enzyme protein in a given preparation and thus to check precisely the enzyme purity and calculate the turnover number of ADH under defined reaction conditions. In 0·1M-NaOH–glycine buffer, pH 10, at 25°C in concentrations of 10 mM ethanol and 0·9 mM NAD^+ the turnover number is $1{\cdot}65 \pm 0{\cdot}15$ of NAD^+ or alcohol molecules converted by one active center of the enzyme per second. The turnover number is low, three times lower than that of the horse enzyme under identical conditions, but it is higher than the turnover number of human enzyme[2].

In Table III Michaelis constants are presented for the two basic coenzyme–substrate pairs, Table IV summarizes Michaelis constants K_m, substrate inhibition constants K_s' and relative maximum reaction rates for some alcohols. In Fig. 5 the pH optimum of pig alcohol dehydrogenase is demonstrated. The K_m values for the sub-

strates are comparable with corresponding values for the horse liver enzyme[1]. Increasing hydrophobicity of the alcohol affects the relative maximum rate of its oxidation only slightly. In this respect the pig enzyme resembles the horse one,

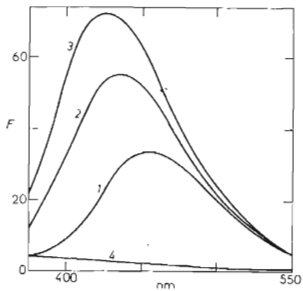

FIG. 3

Fluorescence Emission Spectra of NADH *1*, Binary Complex ADH-NADH *2* and Ternary Complex ADH–NADH–Isobutyramide *3*.

4 Baseline, excitation at 330 nm. Measured in sodium phosphate buffer, pH 7 (*I* 0·1), 6 μM NADH, 1 μM ADH, 0·1M isobutyramide. *F*, relative fluorescence.

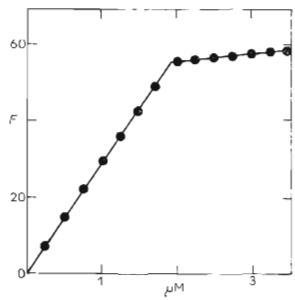

FIG. 4

Direct Fluorescence Titration of Pig Alcohol Dehydrogenase Binding Sites for NADH

Measured in sodium phosphate buffer, pH 7 (*I* 0·1) in the presence of 0·1M isobutyramide. Excitation at 330 nm, emission at 410 nm. The equivalence point (the break in the titration plot) corresponds to 1·95 μM ADH. *F*, relative fluorescence, μM, NADH concentration.

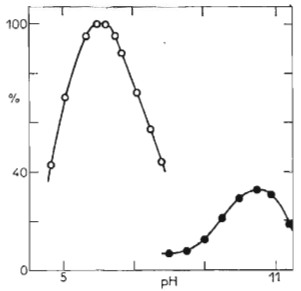

FIG. 5

pH — Optimum of Pig Alcohol Dehydrogenase for the Oxidation of Ethanol ● and for the Reduction of Acetaldehyde ○

Measured at saturation concentrations of substrates and coenzymes. % relative initial enzyme reaction rate, the rate of acetaldehyde reduction at pH 6 corresponding to 100%.

TABLE III

Michaelis Constants of Pig Alcohol Dehydrogenase for Basic Substrates and Coenzymes

Measured at 25°C in sodium phosphate buffer, pH 7, (I 0·1), and 0·7M-NaOH–glycine buffers, pH 10 or pH 10·5, respectively. Enzyme concentration was 0·03 μM.

Variable component	Saturating component mM	K_m, μM		
		pH 7	pH 10	pH 10·5
Ethanol	0·8 NAD	250	420	730
NAD	10·0 ethanol	23	120	120
Acetaldehyde	0·08 NADH	260	—	—
NADH	3·5 acetaldehyde	6	—	—

TABLE IV

Comparison of Michaelis Constants K_m, Substrate Inhibition Constants K_s' and Relative Maximum Rates V_{max} of Pig Alcohol Dehydrogenase for Various Alcohols

Measured at 25°C in 0·07M-NaOH–glycine buffer, pH 10·5, concentration of NAD 1 mM, enzyme concentration 0·03 μM. The maximum rate for ethanol is taken as a unit.

Substrate	K_m, μM	K_s', mM	V_{max}
Ethanol	730	400	1·00
n-Propanol	280	60	1·04
n-Butanol	60	9	0·91
Cyclohexanol	160	10	0·65

TABLE V

Inhibition Constants $K_{0·5}$ for Some Inhibitors of Pig Alcohol Dehydrogenase

Measured at 25°C in sodium phosphate buffer, pH 7 (I 0·1) and in 0·07M-NaOH–glycine buffer, pH 10·5, respectively. 10 mM ethanol, 1 mM-NAD^+, enzyme concentration 0·03 μM.

Inhibitor	$K_{0.5}$, μM	
	pH 7·0	pH 10·5
Isobutyramide	46	40
Pyrazole	—	2
o-Phenantroline	30	43
Berberine	no inhibition	≈3 000
Ethylberberine	> 50	30
Chlorprothixene	>100	9

on the other hand the human enzyme catalyses better the oxidation of more hydrophobic alcohols[11]. In Table V the inhibition constants $K_{0.5}$ are summarized for some inhibitors, which also decrease the activity of other alcohol dehydrogenases[1,10]. The $K_{0.5}$ value equals the concentration of inhibitor that under given conditions decreases the enzyme reaction rate to one half. For testing the inhibitory effect various inhibitors were chosen, which bind to the "substrate pocket" of ADH (ref.[1,10]): *ortho*-phenantroline chelating the active center Zn atom, pyrazole (a strong inhibitor competitive with respect to alcohol), isobutyramide competing with aldehyde, chloroprothixene and berberine alkaloids binding into the substrate pocket apart from the catalytic zinc. It turns out that all inhibitors tested can interact with ADH, but the inhibitory effect of all of them is lower in comparison of the horse liver enzyme[1,10]. The interaction of berberine alkaloids and of chloroprothixene with pig ADH is relatively strong in alcaline and relatively weak in neutral solution. With horse liver ADH an inverted pH effect was found (especially in the case of chlorprothixene)[10].

In spite of some differences in the kinetic or inhibitory behavior the results obtained confirm a substantial similarity of the pig liver alcohol dehydrogenase to other animal alcohol dehydrogenases studied in more detail, especially to the alcohol dehydrogenase from horse liver[1].

REFERENCES

1. Brändén C. I., Jörnvall H., Eklund H., Furugren B. in the book: *The Enzymes* (P. D. Boyer, Ed.), 3rd Ed., p. 103. Academic Press, New York 1975.
2. Pietruszko R.: Advan. Exp. Med. Biol. *56*, 1 (1975).
3. Moser K., Papenberg J., Wartburg J. P. von: Enzymol. Biol. Clin. *9*, 447 (1969).
4. Dalziel K.: Acta Chem. Scand. *12*, 459 (1958).
5. Yonetani T., Theorell H.: Arch. Biochem. Biophys. *99*, 433 (1962).
6. Instruction manual: *Thin-Layer Gel Filtration with the Pharmacia TLG-Apparatus.* Pharmacia 1973.
7. Layne E. in the book: *Methods in Enzymology* (S. P. Colowick, N. O. Kaplan, Eds), p. 451. Academic Press, New York 1957.
8. Lowry O. H., Rosenbrough N. J., Farr A. L., Randall R. J.: J. Biol. Chem. *193*, 265 (1951).
9. Kent R. E., Mc Elvain S. M.: Org. Syn. Coll. *3*, 491 (1955).
10. Kovář J., Skurský L., Protiva M.: This Journal *43*, 2068 (1978).
11. Klyosov A. A., Lange L. G., Sytkowski A. J., Vallee B. L.: Bioorg. Khim. *3*, 1141 (1977).

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