ISOLATION AND CHARACTERIZATION OF ALCOHOL DEHYDROGENASE FROM GERMINATING SUNFLOWER SEEDS

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The characteristics of alcohol dehydrogenase (EC 1.1.1.1) isolated from germinating sunflower seeds are described. After fractionation with ammonium sulfate and chromatography on DEAE-cellulose the specific activity of the enzyme (mol. wt. 60000) increased 62 times. The K_m-value for ethanol is $1{\cdot}5 \cdot 10^{-2}$M and for NAD $0{\cdot}75 \cdot 10^{-4}$M at a pH-optimum of 8·8. The oxidation rate of alcohols decreases from ethanol to hexanol, unsaturated analogs, however, are oxidized faster than saturated analogs. Diols, cyclic alcohols, sugar and terpenic alcohols, and 2-C derivatives of alcohols are not oxidized. The K_m of acetaldehyde reduction is $5{\cdot}6 \cdot 10^{-3}$M at a pH-optimum of 7·2. Amides, oximes, the so-called SH-poisons and chelating agents act as inhibitors. Since the inhibitory effect is decreased after preincubation of the enzyme with NAD and sometimes also with the substrate, the role of SH-groups and of metal atoms in the enzymatic catalysis can be assumed. Many intermediates of the sugar metabolism act as inhibitors not competing with ethanol. The similarity between sunflower ADH and the enzyme from yeast and animal liver is discussed.

Alcohol dehydrogenase has been studied in detail as regards the enzyme from the liver of animals and from yeast[1-3,5-16]. The enzymes isolated from these two sources differ in function: liver alcohol dehydrogenase acts as a catalysts of detoxication of alcohol, *i.e.* of a substance exogeneous for the organism whereas yeast alcohol dehydrogenase catalyses both ethanol synthesis, *e.g.* in the process of glucose degradation under anaerobic conditions *via* transfer of reducing equivalents to acetaldehyde, and also ethanol oxidation to acetate *via* acetaldehyde under aerobic conditions. The enzymes from animals and from yeast differ in molecular weight, stability during the process of isolation, substrate specificity, and subunit composition. The mode of participation of SH-groups in the catalysis as well as the role of zinc atoms, found in both enzymes, have been discussed.

Vegetal alcohol dehydrogenase resembles in function more the yeast enzyme since it catalyses ethanol synthesis during the so-called natural anaerobiosis of the germinating seeds and its oxidation to acetate *via* acetaldehyde after the rupture of the testa.

EXPERIMENTAL

Cultivation of plants: Sunflower (*Helianthus annuus* L.) seeds served as vegetal material. The seeds germinated on a filter paper disc in water in closed Petri dishes 19 cm in diameter. Each dish contained 20 g of dry seeds and 60 ml of water. The germination was allowed to proceed in a thermostated box at 25°C and constant illumination.

Isolation of alcohol dehydrogenase: The germinated seeds were homogenized in a cooled blender with 0·1M phosphate buffer in 0·01M mercaptoethanol, pH 8·5. The homogenate was filtered through double cheesecloth and the filtrate was centrifuged 20 min at 10000 *g* with cooling. The supernatant was again filtered through double cheesecloth and saturated with ammonium sulfate to 35—50% saturation. The sediment was collected by centrifugation 20 min at 10000 *g* with cooling and dissolved in a minimal volume of 0·01M Tris-acetate buffer in 0·01M mercaptoethanol, pH 6·4. The protein fraction obtained by ammonium sulfate fractionation was separated from sulfate ions on a 3 × 45 cm column of Sephadex G-25, equilibrated in 0·01M Tris-acetate buffer in 0·01 mercaptoethanol, pH 6·4. About 150 mg of protein was applied to the column. The column was eluted with 0·01M Tris-acetate buffer in 0·01M mercaptoethanol, pH 6·4 at a flow rate of 1 ml min^{-1} and 5-ml fractions were collected. Ammonium sulfate fractionation was combined with chromatography on a 2·5 × 45 cm column of DEAE-cellulose equilibrated in 0·01M Tris-acetate buffer in 0·01M mercaptoethanol, pH 6·4. About 100 mg of protein was applied to the column. Tris-acetate buffer containing mercaptoethanol, pH 6·4 with increasing concentration of Tris from 0·01 to 0·6M (total volume 1000 ml) was us as an eluent. The flow rate was 0·8 ml/min and 12-ml fractions were collected. The whole operation was carried out in a cold room at 0°C. The separation of the proteins on the DEAE-cellulose column was monitored by measurement of the absorbance of fractions in Spectromom 202 spectrophotometer.

Determination of alcohol dehydrogenase activity: The following solutions were pipetted in the spectrophotometer cell: 0·1 ml of 1M ethanol, 0·06 ml of 7·86 mM-NAD, 0·33 ml of 0·5M phosphate buffer in 0·01M mercaptoethanol, pH 8·5, and 0·41 ml of water. The activity of ADH was measured in Spekol (Zeiss, Jena) spectrophotometer, equipped with an additional amplifier and recorder, in terms of absorbance increase at 366 nm during 2 min after the beginning of the enzymatic reaction. The reaction was started by the addition of 0·1 ml of enzymic preparation diluted so that the absorbance change during 2 min did not exceed the value of 0·1, *i.e.* the measurements were made in a range where the dependence of reaction rate on time is linear. When the activity was examined as a measure of the effect of inhibitors or modulators, 0·1 ml of effector was added to the reaction medium instead of 0·1 ml of water.

Protein content was determined by the method of Lowry and coworkers[19].

Sulfhydryl groups were determined by the method of Sedlak and Lindsay[18]. The method is based on the use of the Ellman reagent (5,5′-dithio-bis-2-nitrobenzoic acid) which gives a yellow reaction product with free sulfhydryl groups.

Molecular weight determinations were carried out by gel filtration on a 1·5 × 40 cm column of Sephadex G-200 as described before[17].

RESULTS AND DISCUSSION

We examined the activity of ADH in germinating sunflower seeds for the first five days of germination and found that this activity reaches a maximum on the first day of germination. We extracted the seeds with sodium phosphate buffer, fractionated

the extract by ammonium sulfate precipitation, and isolated the fraction showing the highest specific activity. This fraction was desalted on Sephadex G-25 and chromatographed on a column of DEAE-cellulose (Fig. 1). This procedure resulted in a 62--fold increase of specific activity (Table I).

We determined the Michaelis constant for ethanol oxidation and for NAD reduction from the plot of reaction rate *versus* substrate concentration. The K_m-value for ethanol is $1{\cdot}5 \cdot 10^{-2}$M and for NAD $0{\cdot}75 \cdot 10^{-4}$M. Sunflower ADH differs in these values approximately by two orders from liver ADH (LADH) and resembles yeast ADH (YADH). We examined the pH-optimum of the sunflower enzyme over the pH-range 4–10 and found that it lies at 8·8.

We have also examined the substrate specificity of sunflower ADH. Methanol was not oxidized; the oxidation rate decreased in the alcohol series from ethanol to hexanol with the increasing number of carbon atoms in the chain. Isopropanol, isobutanol, isoamyl alcohol, and isooctanol, diols, and cyclic alcohols as well as phenylethanol, 2-mercaptoethanol, 2-aminoethanol, benzyl alcohol, glycerol, and cinnamyl alcohol were not attacked (Table II). Sunflower ADH resembles horse LADH because it does not oxidize methanol and oxidizes allyl alcohol faster than ethanol yet, on the other hand, it also resembles YADH since the rate of oxidation of alcohols decreases with the increasing number of carbon atoms in their molecules[3,4]. The enzyme catalyzes also the reduction of acetaldehyde, the pH-optimum of the reaction being 7·2 and the K_m-value $5{\cdot}6 \cdot 10^{-3}$M.

We have also examined the effect of intermediates of sugar metabolism on alcohol oxidation. We found that malate is the most potent inhibitor of sunflower ADH; the enzyme is thus similar to rat LADH (ref.[5]). Acetate and succinate likewise decrease the activity of sunflower ADH and the enzyme differs in this susceptibility

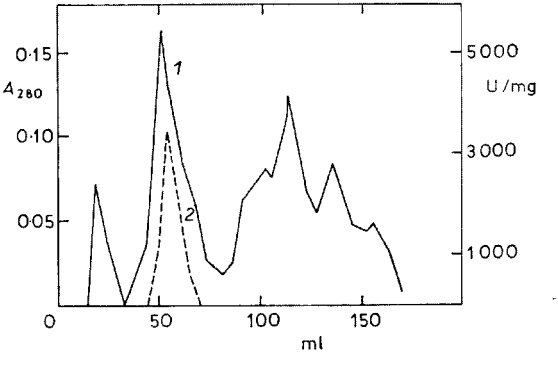

FIG. 1

Separation of Proteins on DEAE-cellulose Column

1 Absorbance at 280 nm, *2* specific activity of ADH (U/mg protein).

TABLE I

Isolation of ADH from Germinating Sunflower Seeds

The values in the Table are related to 10 g of fresh weight of germinating seeds. The specific activity is expressed in units per mg of protein.

Purification degree	Proteins mg	Activity of ADH units . 10^2	Specific activity
Crude extract	135	137·5	102
Fraction precipitated at 35–50% saturation with sulfate	11	75·45	685
Desalted fraction precipitated with sulfate	6·25	47·25	756
Fraction from Chromatography on DEAE-cellulose	0·5	16·2	3 240

TABLE II

Substrate Specificity of Sunflower Alcohol Dehydrogenase

Concentration of substrate 1 . 10^{-2}M.

Substrate	Relative oxidation rate	Substrate	Relative oxidation rate
Ethanol	100	n-Butanol	75
n-Propanol	90	2-Butene-1-ol	82
2-Propene-1-ol	130	n-Hexanol[a]	12

[a] Saturated solution.

TABLE III

Relative Oxidation Rate of Ethanol at 0·1M Concentration by Sunflower Alcohol Dehydrogenase in Presence of 0·1M Solutions of Sugar Intermediates

Substrate	Relative oxidation rate	Substrate	Relative oxidation rate
Ethanol	100	Ethanol + malate	19
Ethanol + lactate	42	Ethanol + succinate	32
Ethanol + pyruvate	85	Ethanol + isocitrate	102
Ethanol + acetate	37		

to acetate and succinate from LADH. Pyruvate, a strong LADH inhibitor, has a minor effect only on ADH, isocitrate has no effect at all. The action of these compounds could play a regulatory role in plants since most substrates showing an effect on the activity of ADH change their concentration during anaerobiosis. None of the metabolites examined competes with ethanol. Unfortunately the action of these substrates as possible modulators of YADH has not been studied so far (Table III).

Of amide inhibitors of LADH we investigated the action on sunflower ADH of acetamide and butyramide and of the oxime inhibitors acetoxime and cyclohexanone oxime. We observed that oximes are stronger inhibitors than amides and that the action of acetamide, butyramide, and cyclohexanone oxime is noncompetitive as

TABLE IV

Effect of Amides and Oximes on Ethanol Oxidation and Acetaldehyde Reduction by Sunflower ADH

The values in the Table show per cent of inhibition. Concentration of inhibitor $1 . 10^{-2}$M.

Inhibitor	Ethanol oxidation	Acetaldehyde reduction
Acetamide	11·0	9·5
Butyramide	12·0	25·0
Acetoxime	37·0	54·0
Cyclohexanone oxime	25·0	65·0

TABLE V

Effect of Inhibitors on Sunflower Alcohol Dehydrogenase

A inhibition without preincubation, B enzyme preincubated with inhibitor, C enzyme preincubated with NAD, D enzyme preincubated with ethanol.

Inhibitor	Concentration mol/l	Inhibition			
		A	B	C	D
Sodium azide	$1 . 10^{-3}$	49	56	39	54
o-Phenanthroline	$1 . 10^{-3}$	47	52	42	41
Salicylaldoxime	$5 . 10^{-4}$	29	43	12	33
α,α′-Dipyridyl	$1 . 10^{-3}$	17	27	10	23
Ethylmaleimide	$1 . 10^{-3}$	17	29	13	19
Cupral	$4 . 10^{-2}$	53	81	53	49
Iodoacetamide	$4 . 10^{-3}$	22	61	21	27

regards ethanol whereas acetoxime acts competitively. The results of the measurement are given in Table IV. Hundred times higher concentrations were necessary compared to LADH to achieve the same effect on the vegetal enzyme[20].

We have also examined the influence of inhibitors binding to the metal component of the enzyme molecule and the effect of products blocking SH-groups. We compared the inhibitory action without preincubation of the inhibitor with the enzyme, after preincubation with the enzyme, the effect on the binary enzyme–coenzyme complex and on the enzyme–substrate complex. Inhibition was increased with all inhibitors after their preincubation with the enzyme; the protective effect of the enzyme–NAD complex or of the enzyme–substrate complex was observed with some inhibitors. The results are given in Table V. The role of SH-groups is considered both in LADH and also in YADH. In the opinion of some authors these groups participate on the binding of the substrate to the binary enzyme–coenzyme complex whereas other authors postulate that these groups play a role in the three-dimensional structure of the enzyme. One of the zinc atoms is supposed to influence the conformation of the enzyme; according to other authors this atom directly participates on the transfer of the hybrid anion. The second zinc atom participates on maintaining the tertiary structure of the protein[6–13].

One of the structural characteristics of sunflower ADH determined in this study is its molecular weight; a value of 60000 was determined in gel filtration experiments. Sunflower ADH thus resembles rat LADH (ref.[5]) yet differs from YADH (ref.[2]).

The total number of sulfhydryl groups found in the molecule of sunflower ADH in preliminary experiments is 6; this value is very low compared to LADH and YADH (ref.[14,15]).

Sunflower ADH resembles LADH in some of its characteristic features such as, *e.g.* the pH-optimum of ethanol oxidation, a higher substrate specificity for allyl alcohol than for ethanol, the susceptibility to the inhibitory effect of malate, and to the effect of amides and oximes, and also the molecular weight. Other properties of the enzyme however, are similar to those of YADH: the values of the Michaelis constants for ethanol and the coenzyme and the decrease of the oxidation rate of alcohols with the increasing number of their carbon atoms. Like YADH and LADH, sunflower ADH also bears metal atoms and sulphydryl groups in its molecule which play a role in catalysis by the enzyme.

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