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Comparative Study of Plant Alcohol Dehydrogenases

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Abstract. Alcohol dehydrogenase was isolated both from monocotyledons and dicotyledons, some of them with proteins (bean, pea), others with lipids (rape, sunflower) and still others with sugars (rice) as reserve substances. Molecular weights of the isolated dehydrogenases ranged from 53 000 to 80 000. Plant alcohol dehydrogenases (ADH) catalyze the oxidation of ethanol as well as the reduction of acetaldehyde. pH optimum for the oxidation is in the alkaline region, for the reduction it is near neutrality. The Michaelis constants for ethanol oxidation are, with the exception of rice, higher than those for reduction of acetaldehyde. The specificity of plant ADH toward alcohols is relatively broad and only quantitatively different in the individual plants. Inhibitors of the ADH's studied are oximes, amides and intermediates of sugar metabolism, such as malate, acetate or succinate. The degree of inhibition brought about by the inhibitors studied differs from plant to plant but the inhibition type is the same.

In a great number of plants, both monocotyledonous and dicotyledonous, containing proteins as reserve substances (bean, lentil, soybean, pea), lipids (rape, sunflower, flax) or starch (maize, barley, oat, wheat, rye) ethanol is produced during the natural anaerobiosis before the rupture of the testa (CAMERON and COSSINS 1967, LOWE and JAMES 1960, LEBLOVÁ *et al.* 1969, LEBLOVÁ *at al.* 1974). After the rupture ethanol disappears. Some authors assume that it is released to the surroundings (FIEDLER 1951, WAGER 1959), our results being in agreement with this assumption. However, ethanol in the cell is also oxidized in a reaction catalyzed by alcohol dehydrogenase to acetylcoenzyme A with acetaldehyde as an intermediate. Acetylcoenzyme A is then further metabolized, *e.g.* in pea through the citric acid cycle (COSSINS and TURNER 1962, CAMERON and COSSINS 1967, PETERSON and COSSINS 1966).

The present paper compares the catalyst of alcohol oxidation as well as of acetaldehyde reduction, by alcohol dehydrogenase (EC 1.1.1.1.) isolated from bean, pea, rape, sunflower and rice.

Materials and Methods

Experiments were done with germinating seeds of the bean (*Phaseolus vulgaris* L., cv. 'Dětenická pnoucí'), pea (*Pisum sativum* L., ssp. *arvense*), rape

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(*Brassica napus* L., cv. 'Třebíčská'), sunflower (*Helianthus annuus* L.) and rice (*Oryza sativa* L. cv. 'Dubovszky 129'), cultivated in amounts of 10 to 20 g on Petri dishes, containing (depending on plant species) 10 to 60 ml deionized water, in a constant-temperature room.

Extraction of Alcohol Dehydrogenase

The enzyme was extracted from seed homogenate after a germination period resulting in maximum activity. A 30—50 % seed homogenate was prepared by grinding of seeds with glass powder and 0.01 M sodium phosphate buffer of pH 8.5, containing 0.01 M mercaptoethanol. The homogenate was then filtered through cheese-cloth and centrifuged at 4 000 *g*, in the case of starch-containing seeds at 10 000 *g*.

Isolation of the Enzyme

The sodium phosphate buffer extract of the enzyme was fractionated with ammonium sulphate, the salted-out fraction was desalted on a Sephadex G-25 column and the eluate was purified chromatographically on DEAE-cellulose as described previously (LEBLOVÁ and STIBOROVÁ 1975), or on CM-cellulose, introducing approximately 20 mg protein in 10—15 ml of desalted solution on a 2 × 30 cm column. Elution was carried out with 0.05 to 0.1 M phosphate (pH 7.5) with 0.01 M mercaptoethanol, the elution volume was 200 ml, the rate of flow 10 ml h^{-1}. Five-ml fractions were collected.

Protein Content

was determined using the technique of LOWRY *et al.* (1951).

Molecular Weight

was estimated by gel filtration on a Sephadex G-200 column (1.6 × 18 cm), equilibrated with 0.01 M Tris-acetate buffer (pH 6.4). Myoglobin (mol. wt. 17 800), ovalbumin (45 000) and gamma globulin (157 000) served as standards.

Results and Discussion

All the plants studied produce ethanol during the first four days of germination, its concentration slowly rising to a maximum and then, after the rupture of the testa, decreasing again. The time course of alcohol dehydrogenase activity is similar. In rape and sunflower the maximum ADH activity

TABLE 1

Specific activity of isolated alcohol dehydrogenases

Plant	Specific activity (units per mg protein)	Degree of purification
Bean	5 620	31 ×
Pea	80 000	135 ×
Rape	4 710	43 ×
Sunflower	12 400	62 ×
Rice	98 394	60 ×

is reached after the first day of germination, in pea and bean after two days and in rice after three days.

Isolation of Alcohol Dehydrogenases

Sodium phosphate extract with ADH activity was precipitated with ammonium sulphate saturated to 35—50 % for bean and sunflower, to 35 to 60 % for rape and to 40—60 % for pea and rice. By salting out with

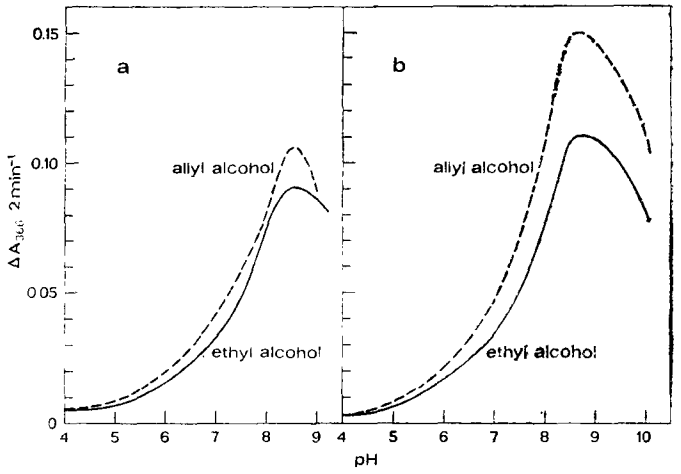

Fig. 1. Activity of ADH from rice (1a) and from pea (1b) as dependent on pH. Abscissa = pH, ordinate = enzymic reaction rate expressed as initial velocity, *i.e.* changes in absorbance at 366 nm during 2 min.

ammonium sulphate the specific activity of the enzyme was increased by an order of magnitude. Desalting on a Sephadex G-25 column was found to be generally useful, resulting in almost all cases in an increase of activity, whereas dialysis led to its loss. The salt-free active preparation was further purified chromatographically. The same degree of purity was obtained with DEAE and CM cellulose. Specific activity of isolated alcohol dehydrogenases

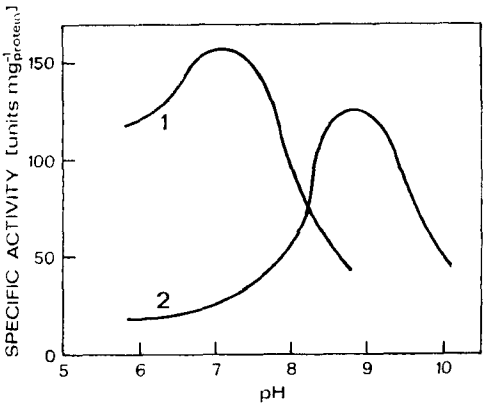

Fig. 2. Dependence of acetaldehyde reduction (curve 1) and ethanol oxidation (curve 2) rates on pH; ADH from rape (for explanation see legend to Fig. 1).

TABLE 2

Michaelis constants for ethanol, allyl alcohol, NAD and acetaldehyde. Molecular weights

ADH from	pH optimum	$K_{m\ ethanol} \times 10^{-2}$ M	$K_{m\ allyl\ alc.} \times 10^{-2}$ M	$K_{m\ NAD} \times 10^{-4}$ M	$K_{m\ acetald.} \times 10^{-2}$ M	Molecular weight
Bean	8.2	3.3	0.8	1.0	1.2	63 000
Pea	8.7	1.8	1.0	0.7	0.7	60 000
Rape	8.8	2.5	2.0	1.0	1.3	63 000
Sunflower	8.8	1.5	1.0	0.8	0.9	53 000
Rice	8.5	0.8	0.7	1.5	1.5	80 000
Yeast[a])		1.4		1.7	2.5	
Liver[a])		0.051		0.13	0.021	

[a]) HAYES and VELICK 1954

TABLE 3

Substrate specificity of alcohol dehydrogenases

Substrate	M	Relative rate of oxidation with ADH from bean	pea	rape	sunflower	rice
Methanol	10^{-2}	0	2	0	0	0
Ethanol	10^{-2}	100	100	100	100	100
n-Propanol	10^{-2}	39	44	60	90	19
2-Propen-1-ol	10^{-2}	171	160	115	130	133
n-Butanol	10^{-2}	19	30	54	75	—
2-Buten-1-ol	10^{-2}	34	49	62	82	—
2-Butanol	10^{-2}	0	0	0	0	0
1,3-Butanediol	10^{-2}	0	0	0	0	0
1,4-Butanediol	10^{-2}	0	0	0	0	7
2-Butene-1,4-diol	10^{-2}	0	0	0	0	0
Isobutyl alcohol	10^{-2}	0	10	—	—	11
Isoamyl alcohol	10^{-2}	0	15	0	5	16
2-Penten-1-ol	10^{-2}	3	6	—	0	24
n-Hexanol	sat.	5	12	25	12	19
Cyclohexanol	sat.	0	0	0	0	0
Isooctyl alcohol	sat.	0	0	0	0	0
Cinnamyl alcohol	sat.	10	16	12	10	—
Mercaptoethanol	10^{-2}	0	3	0	0	0

is shown in Table 1, where the degree of purity with respect to the initial extract in sodium phosphate buffer is also given.

Stability of Enzyme Preparations

The stability of enzyme preparations prepared from seeds of five plant species was followed for three days. It was found that during storage at 0°—4° C the enzyme activity is best preserved in the lyophilized fraction after chromatography whereas the ammonium sulphate fraction is (with the exception of bean and pea) least stable. Lyophilization of fractions obtained after chromatography results in ADH preparations stable for several weeks.

The alcohol dehydrogenases studied were heat-labile. When heated to 60—65 °C their activity is lost completely. Stability is not increased by addition of NAD; on the contrary, the enzyme is labilized in a 0.5 mM NAD solution. 1 M ethanol shows a slightly protective action.

Properties of Plant Dehydrogenases

1. *pH Optimum*

The rate of ethanol oxidation was studied between pH 4 and 10 in Tris acetate or phosphate buffers, the pH optimum being in the alkaline region (Table 2). It is identical for the oxidation of both ethanol and allyl alcohol (Fig. 1). The rate of acetaldehyde reduction is highest at about pH 7 (Fig. 2).

2. *Michaelis Constants*

a) Oxidation of substrate. The dependence of the oxidation rate on the concentration of both the substrate and of the coenzyme was studied and Michaelis constants for ethanol and allyl alcohol in the range from 5×10^{-2}

TABLE 4

Effect of metabolic intermediates on ethanol oxidation rate. The figures indicate relative rates of oxidation, the control with ethanol being set equal to 100

ADH from	lactate	malate	pyruvate	acetate	succinate
Bean	49	22	72	40	29
Pea	16	5	28	17	31
Rape	38	26	51	36	25
Sunflower	42	19	85	37	32
Rice	19	6	73	13	29
Liver	99	0	0	100	125

to 1×10^{-3} M and for NAD in the range from 5×10^{-3} to 1×10^{-5} M were determined by Lineweaver's method. The K_m's for ethanol at the pH optimum are of the same order of magnitude (10^{-2} M); for allyl alcohol they are only slightly lower. The K_m's for NAD are similar for all ADH's, of the order of 10^{-4} M (Table 2).

b) Reduction of substrate. The enzyme catalyzes not only ethanol oxidation but also acetaldehyde reduction. The K_m's for acetaldehyde reduction lie between 10^{-2} and 10^{-3} M. With the exception of rice, all the ADH's studied display a lower K_m constant for aceta ldehyde reduction than for ethanol oxidation (Table 2). The K_m's for NADH are 10^{-4} to 10^{-5} M.

3. *Substrate Specificity*

The ADH catalyzes the oxidation of a number of alcohols (Table 3). Aqueous solutions of alcohols at the concentrations shown in the table were used; with poorly soluble substrates saturated aqueous solutions were applied. Quantitative rather than qualitative differences in substrate specificity were found between individual alcohol dehydrogenases. Methanol was oxidized only very slightly and only by the enzyme from pea, 2-alken-1-ols were oxidized faster than the saturated homologues. None of the enzymes catalyzes oxidation of diols, sugar polyols and cyclic alcohols. Neither 2-methoxy-, nor 2-aminoethanol served as a substrate. Isooctyl alcohol was not metabolized and isoamyl alcohol as well as isopropyl alcohol were oxidized only imper-

TABLE 5

Effect of amides and oximes on acetaldehyde reduction (A) and ethanol oxidation (B). Percent inhibition with 10^{-1} M substrate and 10^{-2} M amide or oxime concentration

Inhibitor	ADH from bean		pea		rape		sunflower		rice	
	A	B	A	B	A	B	A	B	A	B
Butyramide	0	0	19	5	2	0	25	12	7	6
Acetoxime	28	24	64	21	41	28	54	37	1	10
Cyclohexanone oxime	21	10	73	16	30	19	65	25	13	1
Acetamide	0	4	5	5	8	2	10	11	6	7

ceptibly. As a general rule, the rate of oxidation of alcohols decreases with the increasing number of carbon atoms in the molecule.

4. *Inhibitors of Alcohol Dehydrogenases*

a) Metabolic intermediates. The effects of the applied intermediates as modulators of animal alcohol dehydrogenase are shown in Table 4. All were used at a concentration of 0.1 M and all of them proved to be more or less efficient inhibitors of plant ADH. The inhibition was noncompetitive with ethanol for all substances and all the five ADH's tested. The liver enzyme is not inhibited by all substances (ARSLANIAN *et al.* 1971): Pyruvate and malate are potent inhibitors, whereas lactate and acetate do not affect the enzyme activity and succinate has a stimulatory effect.

b) Effect of amides and oximes. Acetamide and butyramide were used as representatives of amides, acetoxime and cyclohexanonoxime as oximes. Inhibitions induced in the individual plant species are given in percentages in Table 5. In general, oximes are seen to inhibit ADH activity more than amides, the reduction of acetaldehyde being inhibited more than the oxidation of ethanol. Acetoxime competes with the substrate, the action of acetamide, butyramide and cyclohexanonoxime is noncompetitive.

5. *Molecular Weights of Plant Dehydrogenases*

Molecular weights estimated by gel filtration are given in Table 2. So far the molecular weight has been known only for the enzyme from pea where, in agreement with our results, it was found to be 60 000 (COSSINS *et al.* 1968) and for tea and peanut, where it was over 100 000 (PATTEE and SWAISGOOD 1968, HATANAKA *et al.* 1974). The molecular weights of the enzymes from human, rat and horse liver range from 60 000 to 80 000, the yeast enzyme has a molecular weight of 150 000 (BODNEY and BLAIR 1971, ARSLANIAN *et al.* 1971, HAYES and VELICK 1954, JÖRNVAL 1970).

It may be concluded that plant dehydrogenases isolated from bean and pea seeds with prevalence of proteins, from rape and sunflower seeds with storage lipids and from rice seeds rich in starch, differ in the protein moiety of their molecules; the conclusion is supported by the different solubility of these proteins and by different molecular size, even if their molecular weights are fairly similar. All the enzymes studied catalyze both the oxidation of ethanol (with the same dependence and K_m) and the reduction of acetaldehyde. Unsaturated alcohol analogues are oxidized more avidly than the saturated ones, as testified by the comparison of K_m for ethanol and allyl alcohol. Differences in substrate specificity toward alcohols are quantitative rather than qualitative. The coenzyme of plant dehydrogenases is NAD and the behaviour of the individual enzymes to this cosubstrate are analogous. The degree of inhibition brought about by oximes and amides is different and the same applies to metabolites, such as lactate, malate, succinate, pyruvate and acetate, the concentrations of which in plant tissues vary during anaerobiosis and may thus play a regulatory role (STREETER and THOMPSON 1972a,b MCMANMON and CRAWFORD 1971, MAZELIS and VENNESLAND 1957). The plant enzymes are different (with respect to their molecular weights, Michaelis constants, substrate specificity and effects of inhibitors) from the yeast enzyme as well as from the widely studied enzyme of mammalian liver; they

represent a separate group of enzymes with certain differences between individual plant species.

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