RICE AND PEA ALCOHOL DEHYDROGENASE

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We have isolated and partly characterized the alcohol dehydrogenase (ADH) from germinating seeds of pea, in which proteins prevail as stock products, and of rice with starch as the main stock product. We used for the isolation fractionation with ammonium sulfate, desalting on a column of Sephadex G-25, and chromatography on DEAE-cellulose. The specific activity of ADH isolated by this method from the germinating seeds of pea and rice was 135- and 60-times, respectively higher than the activity of the crude extracts. The pH-optimum of both pea ADH and rice ADH lies in the slightly alkaline region; the K_m-value with respect to ethanol is $1\cdot78 . 10^{-2}$M (the pea enzyme) and $0\cdot78 . 10^{-2}$M (the rice enzyme), with respect to acetaldehyde $0\cdot67 . 10^{-2}$M (pea enzyme) and $1\cdot54 . 10^{-2}$M (rice enzyme). We have assayed the effect of a number of inhibitors and intermediates of the sugar metabolism on the activity of both ADH's studied. The molecular weight of pea and rice ADH is 60000 and 80000, respectively. Both enzymes are very similar yet not identical. It appears that the alcohol dehydrogenases from plants represent a group of alcohol dehydrogenases differing both from ADH's isolated from the liver of mammals and also from yeast ADH.

Alcohol dehydrogenase (E.C.1.1.1.1), an enzyme involved in the ethanol metabolism, is present in a number of germinating seeds during the period of natural anaerobiosis[1-29], *i.e.* before the rupture of the tests. Similarly to many other enzymes, the efforts to purify and characterize the ADH's from plants have been so far less successful than the experiments with the animal enzymes.

In the present study we have made an attempt to isolate and characterize alcohol dehydrogenase from pea and rice seeds. We were interested in a comparison of the properties of the enzymes from these two plants which differ in (*a*) the kind of their stock products (proteins prevail in pea and starch in rice), (*b*) manner of cultivation (rice grows *de facto* under anaerobic conditions,) which most likely is responsible for the fact that, (*c*) rice ADH is induced by ethanol[9] whereas the activity of pea ADH depends on the endogeneous concentration of both ethanol and acetaldehyde[30].

EXPERIMENTAL

Extraction of enzyme. Pea (*Pisum sativum* I, ssp. *arvense*) and rice (*Oryza sativa*, cv. *Dubovszky* 129) seeds were used in our experiments; the germinating period was chosen so that the specific activity

of ADH be maximum. The germinating seeds were homogenized with glass powder in 0·01M phosphate buffer containing 0·01M 2-mercaptoethanol, pH 8·5. The homogenate was filtered through gauze and the filtrate was centrifuged 20 min at 4000 *g* (pea) or 10000 *g* (rice).

Purification of enzyme. The extract obtained was fractionated with ammonium sulfate. The fraction showing the highest activity was desalted on a column of Sephadex G-25 (dimensions 2×40 cm, load 150 mg of protein at the most). The elution was effected by 0·01M Tris-acetate buffer containing 0·01M 2-mercaproethanol, pH 6·4.

The active fractions were chromatographed on DEAE-cellulose (column dimensions 2·5×30 cm, load 120 mg of proteins at the most). The elution was effected by Tris-acetate buffer containing 0·01M 2-mercaptoethanol, pH 6·4; a linear concentration gradient of Tris (0·01M to 0·6M, total volume 1000 ml) was used[4-6]. The active fractions (volume 5 ml) were pooled, concentrated by lyophilization, and used for the determination of the properties of the enzymes; for this purpose the fractions were dissolved in a minimal volume of 0·01M Tris-acetate buffer containing 0·01M 2-mercaptoethanol, pH 6·4. All operations were carried out in a cold room.

Determination of enzymatic activity. The assay was made in a Spekol spectrophotometer cell into which were pipetted 0·1 ml of 1M ethanol, 0·06 ml of 7·86 mM NAD, 0·33 ml of 0·5M sodium phosphate buffer, pH 8·5, and 0·41 ml of water. The reaction was initiated by the addition of 0·1 ml of the enzyme preparation to the reaction medium. The activity of the enzyme was determined in terms of absorbance increase at 366 nm for 2 min from the beginning of the reaction. One enzyme unit is defined as the quantity of enzyme which brings about a change of 0·001 in absorbance (ref.[31,32]).

The concentration of proteins was determined by the method of Lowry and coworkers[33] after the removal of Tris by dialysis against water.

The determination of molecular weight was carried out by gel filtration on a 1·6×18 cm column of Sephadex G-200. The proteins were eluted by 0·01M Tris-acetate buffer, pH 6·4, at a rate of 3 ml per 20 min. Proteins of known molecular weight were used as standards: albumin (mol. wt. 67000), hemoglobin (64500), ovalbumin (45000), myoglobin (17800), γ-globulin (157000), and blue dextran (to determine the void volume of the column). The molecular weights were established by determination of the elution volumes; the molecular weights were read off a V_e *versus* log mol. wt. plot which is linear for Sephadex G-200 in the molecular weight range 10000–180000 (refs[34,35]).

RESULTS AND DISCUSSION

Purification of ADH *from Pea and Rice Seeds*

The activity of ADH in pea and rice seeds increases during the first hours of germination up to a maximum which is attained after 2 or 3 days of germination (pea and rice, respectively) (Fig. 1). When we fractionated the extract with ammonium sulfate, we found the highest activity of both pea and rice ADH in the fraction precipitated at 40–60% saturation with ammonium sulfate. The specific activity of pea and rice ADH increased 135- and 60-times respectively compared to the crude extract after fractionation with ammonium sulfate and chromatography on DEAE-cellulose. Rice ADH of specific activity 98 400 units per mg of protein was more active (Table I).

TABLE I
Purification of ADH from Pea and Rice Seeds (H-data on pea ADH, R-data on rice ADH)

Fraction	Total activity units		Total protein mg		Specific activity units/mg	
	H	R	H	R	H	R
Extract	3 700 000	457 000	5 957	277	589	1 650
40–60% Saturation with ammonium sulfate	3 250 000	415 800	570	52	4 516	8 153
Effluent from Sephadex G-25 column	2 475 000	291 620	357	28	6 962	11 415
Effluent from DEAE-cellulose column	1 755 000	88 546	24	0·9	74 800	98 384

Properties of Pea and Rice ADH

The Michaelis constants were determined by the method of Lineweaver and Burke. These values differ only little as regards NAD as coenzyme: $K_m = 1\cdot49 . 10^{-4}$M (pH 8·6, pea ADH), $K_m = 1\cdot07 . 10^{-4}$M (pH 8·5, rice ADH). Interest deserves the difference in K_m with respect to oxidation of ethanol and reduction of acetaldehyde, The values measured at pH 8·5 permit us to conclude that pea ADH reduces acetaldehyde at a higher rate and, on the contrary, that ethanol is a better substrate for rice ADH.

enzyme	K_m for oxidation of ethanol	K_m for reduction of acetaldehyde
pea ADH	$1\cdot78 . 10^{-2}$M	$0\cdot67 . 10^{-2}$M
rice ADH	$0\cdot78 . 10^{-2}$M	$1\cdot54 . 10^{-2}$M

The pH-optimum of pea ADH is 8·6 and of rice ADH 8·5, both for ethanol and allyl alcohol as substrates.

The substrate specificity was examined with a number of alcohols. Both ADH's left unattacked sec-butanol, isooctanol, 1,3-butanediol, 2-butene-1,4-diol, cyclohexanol, colamine and phenyl ethanol, methoxyethanol diethylene glycol. Moreover,

1,4-butanediol is not a substrate for pea ADH and the rice ADH does not oxidize ethoxyethanol, methanol and mercaptoethanol. We used also in addition to the alcohols certain metabolic intermediates as substrates (isocitrate, pyruvate, lactate, acetate, malate, and succinate). The rate of oxidation of these compounds by both enzymes equals approximately one tenth of the rate of oxidation of ethanol.

Both ADH's differ in substrate specificity: the relative rates of oxidation of some substrates are different (Table II), methanol and mercaptoethanol are oxidized (albeit only slightly) by the pea enzyme only. Pea ADH resembles yeast ADH in the fact that methanol is its substrate that the activity of ADH toward butanol as substrate is lower than the activity toward ethanol and that it does not oxidize cyclohexanol. The activity of rice ADH toward ethanol is higher than toward butanol and cyclohexanol is not oxidized at all; rice ADH unlike pea ADH, however, does not oxidize methanol and resembles liver ADH in this respect.

A fact deserving interest is the action of intermediates of the sugar metabolism several of which could participate *in vivo* on the reoxidation of NADH (ref.[36]) and on the oxidation of ethanol. For reasons of comparison, we present in Table III also the values measured with rat liver ADH (ref.[37]). Malate of all the intermediates tested acts as the strongest inhibitor of ethanol oxidation by all three enzymes. The action of pyruvate is different: it inhibits rice ADH to 30% only, pea ADH to 70%, and liver ADH to 100%. By contrast, acetate and lactate, which are without effect on animal ADH, inhibit the plant enzymes. All these metabolites are noncompetitive

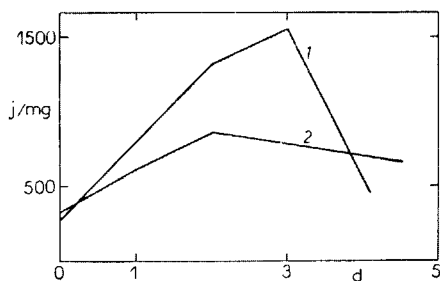

FIG. 1

Specific Activity of Pea and Rice ADH during Germination

d Germination period in days, u/mg activity of ADH in units per mg of protein. *1* rice ADH, *2* pea ADH.

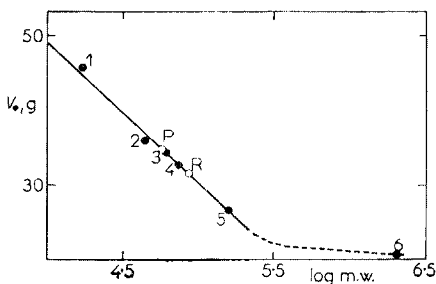

FIG. 2

Determination of Molecular Weight of Pea and Rice ADH by Gel Filtration on Sephadex G-200

1 Myoglobin, 2 ovalbumin, 3 hemoglobin, 4 albumin, 5 γ-globulin, 6 blue dextran; P pea ADH, R rice ADH; V_e, (g) elution volume of the protein determined by weighing and given in grams.

TABLE II

Substrate Specificity of Pea and Rice ADH

Substrate	Substrate concentration M	Relative oxidation rate	
		rice ADH	pea ADH
Ethanol	10^{-2}	100	100
2-Mercaptoethanol	10^{-2}	0	9·4
Methanol	10^{-2}	0	7·5
Propanol	10^{-2}	19	26
Butane-1,4-diol	10^{-2}	7	0
Isobutyl alcohol	saturated aq. solution	11	18
Isoamyl alcohol	saturated aq. solution	16	29
Pent-4-en-1-ol	saturated aq. solution	24	17
Hexanol	saturated aq. solution	19	22
Allyl alcohol	10^{-2}	133	132
Isocitrate	10^{-1}	9·3	9·7
Pyruvate	10^{-1}	23	16
Lactate	10^{-1}	14	11
Acetate	10^{-1}	8·6	8·7
Malate	10^{-1}	14·3	13·2
Succinate	10^{-1}	11·5	15·4

TABLE III

Effect of Some Metabolites on Rate of Oxidation by Liver, Pea, and Rice ADH

The number in the Table stands for the relative rate of oxidation of the substrates. Concentration of ethanol 0·1M, concentration of metabolites 0·2M.

Substrate	Pea ADH	Rice ADH	Liver ADH
Ethanol	100	100	100
Ethanol + lactate	16	19	99
Ethanol + pyruvate	28	73	0
Ethanol + succinate	31	29	125
Ethanol + malate	5	6	0
Ethanol + acetate	17	13	100
Ethanol + isocitrate	105	102	unmeasured

as regards ethanol (the inhibition constants are given in Table VII). The action of the intermediates of the sugar metabolism has not been explained so far even with

rat liver ADH. Our data show that plant ADH's are different from animal ADH's and that they differ quantitatively as regards the effect of inhibitors and also among each other.

TABLE IV

Effect of Some Inhibitors on Oxidation of Ethanol by Pea and Rice ADH

The values in the Table indicate the % of inhibition. I + S + NAD: effect of inhibitor without preceding preincubation of the enzyme with the substrate or NAD; I + E: enzyme preincubated 5 min with the inhibitor; E + NAD: enzyme preincubated 5 min with NAD; E + S: enzyme preincubated 5 min with ethanol.

Inhibitor	Inhibitor Conc. mol/l	I + S + + NAD	I + E	E + NAD	E + S
		pea enzyme			
N-Ethylmaleimide	10^{-3}	13	19	10	10
Sodium azide	10^{-3}	66	69	65	69
α,α'-Dipyridyl	10^{-3}	58	67	48	60
o-Phenanthroline	10^{-3}	58	58	55	56
Cupral	$4 . 10^{-2}$	55	73	50	56
Ferron	$5 . 10^{-4}$	77	83	63	83
Salicylaldoxime	$5 . 10^{-4}$	80	80·5	73	83
		rice enzyme			
N-Ethylmaleimide	10^{-3}	41	53	53	49
Sodium azide	10^{-3}	72	76	66	77
α,α'-Dipyridyl	10^{-3}	75	85	71	32
o-Phenanthroline	10^{-3}	24	43	21	25
Cupral	$4 . 10^{-2}$	18	50	26	15
Ferron	$5 . 10^{-4}$	68	82	58	67
Salicylaldoxime	$5 . 10^{-4}$	40	58	60	75

The inhibition of both plant ADH's by N-ethylmaleimide and the partial protection of the activity of the enzyme by sulfhydryl reagents (2-mercaptoethanol) show that the SH-groups are essential for enzymatic activity. The inhibition by chelating agents (sodium azide, α,α'-dipyridyl, *o*-phenanthroline, ferron) indicates that the enzyme molecules contain a metal component important for their activity (the metal component of the animal enzyme is zinc[38-40]). We found that the preincubation of the enzyme with NAD has a protective effect against all these reagents (Table IV). This demonstrates that the metal component is important for the binding of the coenzyme.

Some amides and oximes act predominantly as inhibitors of the oxidation of acet-aldehyde by animal ADH's (this explains their use in the treatment of alcoholism[41-43]) yet they also inhibit the oxidation of ethanol catalyzed by liver ADH (ref.[42]). These compounds are less effective on pea and rice ADH compared to the liver enzyme. A concentration of amides and oximes by almost 4 orders higher compared to liver

TABLE V

Effect of Amides and Oximes on Oxidation of Ethanol by Pea and Rice ADH

The values in the Table indicate the % of inhibition. I + S + NAD: effect of inhibitor without preceding preincubation of the enzyme with the substrate or NAD; I + E: enzyme preincubated 5 min with the inhibitor; E + NAD: enzyme preincubated 5 min with NAD; E + S: enzym preincubated 5 min with ethanol.

Inhibitor	Inhibitor conc. mol/l	I + S + + NAD	E + I	E + NAD	E + S
		pea enzyme			
Acetamide	10^{-1}	48·3	50	41	46·5
Butyramide	10^{-1}	37	59	7	37
Acetoxime	$3 . 10^{-2}$	75	79·7	73	71·5
Cyclohexanonoxime	$4 . 10^{-2}$	64	76·8	61	72
		rice enzyme			
Acetoxime	10^{-1}	30·2	56·6	59	54·7
Cyclohexanonoxime	$3 . 10^{-2}$	76·5	82	71	65

TABLE VI

Effect of Amides and Oximes on Reduction of Acetaldehyde

The values in the Table indicate the % of inhibition. Concentration of inhibitors 10^{-2}M concentration of ethanol and acetaldehyde 10^{-1}M.

Inhibitor	Pea ADH		Rice ADH	
	oxidation of ethanol	reduction of acetaldehyde	oxidation of ethanol	reduction of acetaldehyde
Acetamide	5·3	4·5	6·7	5·8
Butyramide	5·3	18·8	6	6·8
Acetoxime	21·3	63·5	10	1
Cyclohexanonoxime	16	73	0·5	13

TABLE VII

Inhibition Constants Characterizing Effect of Various Inhibitor Types on Oxidation of Ethanol by Pea and Rice ADH

Inhibitor	Inhibition constant, mol/l	
	pea ADH	rice ADH
Pyruvate	$8·72 . 10^{-2}$	$6·33 . 10^{-2}$
Lactate	$9·09 . 10^{-2}$	$8·44 . 10^{-2}$
Succinate	$6·01 . 10^{-2}$	$9·05 . 10^{-2}$
Malate	$6·54 . 10^{-2}$	$8·46 . 10^{-2}$
Acetamide	$4·00 . 10^{-2}$	no inhibition
Butyramide	$4·54 . 10^{-2}$	no inhibition
Acetoxime	$0·30 . 10^{-2}$	$0·31 . 10^{-2}$
Cyclohexanonoxime	$0·33 . 10^{-2}$	$1·22 . 10^{-2}$
Dimethyl sulfoxide	$4·60 . 10^{-2}$	$3·50 . 10^{-2}$

ADH must be used[44]. As regards pea ADH, acetoxime competes with ethanol whereas acetamide, butyramide, and cyclohexanonoxime act noncompetitively. Oximes only are effective inhibitors of rice ADH. Acetoxime is competitive and cyclohexanonoxime noncompetitive as regards ethanol. Preincubation of the enzymes with NAD is a protection against the action of certain amides and oximes. It is interesting that the oximes decrease the rate of reduction of acetaldehyde by pea ADH 3-times more than the rate of oxidation of ethanol, whereas neither amides nor oximes affect the rate of reduction of acetaldehyde by rice ADH (Table V and VI).

It has been observed in experiments on animals that dimethyl sulfoxide is equally hepatotoxic for animals as for humans. Since dimethyl sulfoxide can compete with acetaldehyde, it can condition the toxic effects of ethanol under certain circumstances. It has not been shown as yet whether the biological activity of dimethyl sulfoxide underlies only the inhibition of liver ADH (ref.[45]). Dimethyl sulfoxide inhibits the oxidation of ethanol by pea and rice ADH noncompetitively with ethanol. The inhibition constants given in Table VII were determined by the method of Dixon[46]. Their order does not differ from the order of the K_i-values found with liver ADH (ref.[45]).

We found in gel filtration experiments on Sephadex G-200 that the molecular weight of pea ADH is 60 000 ± 5 000 and the molecular weight of rice ADH 80 000 ± 5 000 (Fig. 2).

The comparison of the properties of pea and rice ADH with the properties of liver and yeast ADH (Michaelis constants, pH-optimum, substrate specificity, molecular weight) and the observed action of various types of inhibitor and intermediates of

sugar metabolism lead us to conclude that (*a*) the plant ADH's represent an additional group of alcohol dehydrogenases, which resemble more animal alcohol dehydrogenases in some respects and yeast alcohol dehydrogenases in other respects, and (*b*) that alcohol dehydrogenases from individual plant species moreover differ from each other.

REFERENCES

1. Cossins E. A., Turner E. R.: Ann. Bot. (London) *26*, 591 (1962).
2. Sládková J.: *Diploma Thesis*. Charles University, Prague 1971.
3. Leblová S., Mančal P., Sofrová D., Barthová J.: Biol. Plant. *15*, 405 (1973).
4. Suzuki Y., Kyuwa K.: Physiol. Plant. *27*, 121 (1972).
5. Suzuki Y.: Phytochemistry *5*, 761 (1966).
6. Eriksson A. E.: Acta Chem. Scand. *21*, 304 (1967).
7. Cameron D. S., Cossins E. A.: Biochem. J. *105*, 323 (1967).
8. Phillips P.: Amer. J. Bot. *34*, 62 (1947).
9. App A. A., Meiss A. N.: Arch. Biochem. Biophys. *77*, 181 (1958).
10. Sládková J.: *Thesis*. Charles University, Prague 1972.
11. Tučková E.: *Thesis*. Charles University, Prague 1972.
12. Duffus J. H.: Phytochemistry *7*, 1135 (1968).
13. Zimáková I.: *Thesis*. Charles University, Prague 1970
14. Pattee H. E., Swaisgood H. E.: J. Food Sci. *33*, 250 (1968).
15. Leblová S., Ehlichová D.: Phytochemistry *11*, 1345 (1972).
16. Leblová S., Zimáková I., Barthová J., Ehlichová D.: Biol. Plant. *13*, 33 (1971).
17. Kolöffel C.: Acta Bot. Nederl. *18*, 406 (1969).
18. Hatanaka A., Harada T.: Agr. Biol. Chem. *36*, 2033 (1972).
19. Stafford A. H., Vennesland B.: Arch. Biochem. Biophys. *44*, 404 (1953).
20. Arnon D. I.: Science *116*, 635 (1952).
21. Bartels H.: Planta *55*, 583 (1960).
22. Davies D. D., Ugochukwu E. N., Patil K. D., Towers G. H. N.: Phytochemistry *12*, 523 (1973) and *12*, 531 (1973).
23. Efron Y.: Science *170*, 75 (1970).
24. Efron Y., Peleg M., Ashri A. A.: Biochem. Genet. *9*, 299 (1973).
25. Eriksson A. E.: J. Food Sci. *33*, 250 (1968).
26. Goksöyr J., Boeri E., Bonnichsen R. K.: Acta Chem. Scand. *7*, 657 (1953).
27. Hageman R. H., Flesher D.: Arch. Biochem. Biophys. *87*, 203 (1960).
28. Peterson A., Cossins F. A.: Can. J. Biochem. *44*, 423 (1966).
29. Scandalios J. G.: Science *166*, 623 (1969).
30. Kolöffel C.: Acta Bot. Nederl. *17*, 79 (1968).
31. Cossins H. A., Kopala L. C., Blawacki B., Spronk A. M.: Phytochemistry *7*, 1125 (1968).
32. Racker E.: J. Biol. Chem. *184*, 313 (1950).
33. Lowry H. O., Rosenbrough N. J., Farr A. L., Randall R. J.: J. Biol. Chem. *193*, 265 (1951).
34. Andrews P.: Biochem. J. *91*, 222 (1964).
35. Whitaker J. R.: Anal. Chem. *35*, 1950 (1953).
36. Pawan G. L. S.: Nature *220*, 374 (1968).
37. Arslanian M. J., Pascoe E., Reinhold J. G.: Biochem. J. *125*, 1039 (1971).
38. Pho D. B., Bathuna J. L.: Biochem. Biophys. Res. Com. *47*, 419 (1972).
39. Blomquist Ch. H., Smith D. A., Martinez A. M.: Arch. Biochem. Biophys. *112*, 248 (1967).

40. Shore J. D., Theorell H.: Eur. J. Biochem. *2*, 32 (1967).
41. Lester D.: Science *169*, 282 (1970).
42. Nachman M., Lester D., Le Magnen J.: Science *168*, 1244 (1970).
43. Theorell H.: Experientia *21*, 553 (1965).
44. Koe B. K., Tenen S. S.: Fed. Proc., Fed. Amer. Soc. Exp. Biol. *28*, 546 (1969).
45. Perlman R. L., Wolf J.: Science *160*, 317 (1968).
46. Dixon M., Webb E. C.: *Enzymes* IV. Longmans & Co, London 1964.

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