BIOLOGIA PLANTARUM (PRAHA)

13 (1) : 33—42, 1971

On Plant Alcohol Dehydrogenases

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Received January 25, 1970

Abstract. We have found in a number of plants (lentil, lupine, bean ,barley, oats, rye, wheat, cucumber, melon, flax, sunflower and rape) that varying amounts of ethanol are formed under natural anaerobiosis and, that in later growth periods these plants continue to react to anaerobiosis by formation of ethanol. When the testa has opened in germinating plants or, when plants are transferred from the anaerobic atmosphere to air, ethanol disappears.

Plants contain alcohol dehydrogenases, the activity of which depends on the alcohol concentration in their tissue; the maximum concentration is reached during natural anaerobiosis, rising in the course of further growth when the plants are kept in a nitrogen atmosphere.

Alcohol dehydrogenases of the plants studied are localised in the soluble cell fraction not-sedimenting at 120 000 g, their pH optimum is in the weakly alkaline region and their Michaelis constants are equal in order of magnitude (10^{-5} M). They are all inhibited in the same way by Zn^{2+}, Cu^{2+}, Hg^{2+}, $B_4O_7^{2-}$ ions, p-chloromercuric benzoate, iodoacetate, EDTA and phenantroline, which may be considered as evidence of the presence of —SH groups. The specific activity of alcohol dehydrogenase preparations is higher in plants grown in light than in plants grown in the dark.

The specific activity of plant alcohol dehydrogenases can be increased by precipitation with ammonium sulphate by at most one order of magnitude, while all the activity is lost by this purification process in the case of cereals.

The following isoenzyme composition of ADH was found by means of electrophoresis on polyacrylamide: the enzyme from peas and sunflower, for example, is composed of three, that from wheat and oats six, the enzyme from maize and barley of five isoenzymes.

While animals when transferred into anaerobic conditions die in fact due to cell hyperacidification, plants are able to emit carbon dioxide for some time. Some plants can live for rather a long time without oxygen, several days or even weeks; other plants do not survive more than a few hours. All plants in fact pass through a period of natural anaerobiosis during germination, until their testa breaks.

The possiblity of living under anaerobic conditions is related

a) to the capacity of obtaining sufficient energy by processes taking place in the absence of air, *i.e.* fermentation. There exist some plants which survive longer in nitrogen when ATP is added, while with other plants no positive effect was recorded (SIEGEL and ROSEN 1962).

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b) to tolerance to the products of anaerobiosis or, perhaps, the capacitiy of degrading (detoxicating) them.

Under anaerobic conditions pyruvic acid converts either to acetaldehyde and ethanol or to lactic acid. Up to the present, no other products of anaerobiosis have been proved in plants. Plants degrade the ethanol formed under anaerobic conditions when exposed to air. In spite of the large number of papers published in this field, the degradation mechanism is not as yet completely understood. Some authors believe that ethanol is removed not by means of metabolism, but by respiration (WAGER 1959, PHILIPS 1947, KENEFICK 1962, FIDLER 1951). Other authors have found that ethanol labelled with radiocarbon on C-2 converts to acetaldehyde, and further to glutamate and asparagate, while C-1 labelling leads to the formation of labelled amino acids and small amounts of CO_2 with radioactive carbon. (CASTELFRANCO *et al* 1963. LIU *et al.* 1965, PETERSON and COSSINS 1966, CAMERON and COSSINS 1967, COSSINS 1962). Very little is known about the nature of the enzyme which is capable of oxidizing ethanol in plants.

In this paper we have studied the formation of ethanol in plants with different reserve substances under natural as well as artificial anaerobiosis. We have proved that all the plants studied contained alcohol dehydrogenase. We are trying to purify the enzyme and to characterize it in greater detail.

Material and Methods

Plant Cultivation

The experimental plants [lentils (*Leus esculenta* MOENCH cv. Hrotovická), lupine (*Lupinus albus*), peas (*Pisum sativum* cv. Orlík and *Pisum sativum* I. ssp. arvense (L.)), beans (*Phaseolus vulgaris* L. cv. Veltruská Saxa), maize (*Zea mays* L.), barley (*Hordeum distichon* L. cv. Valtický), oats (*Avena sativa* L.), rye (*Secale cereale* L.), wheat (*Triticum aestivum* L. s.s.), cucumber (*Cucumis sativus* L.), melon (*Cucumis melo* L.), sunflower (*Helianthus annuus* L. cv. Slovenská sivá), flax (*Linum usitatissimum* L.), rape (*Brassica napus* L. var. *arvensis* (LAM) THELL.)]* were cultivated in Petri dishes for the short-term experiments. We distributed 7 g seeds over the bottom of a 12 cm diameter dish and added 30 g water. For long-term experiments we removed the testa from the seeds on the third day of germination and transplanted the seeds into polyethylene dishes (135 × 200 mm) in which gauze was stretched on a glass frame above the bottom of the dish. We cultivated the plants in Knop's nutritive solution which we exchanged every day, so that disinfectants did not have to be added even with prolonged cultivation times. When cultivating under anaerobic conditions we placed

* Seeds, the variety of which is not stated, were conventional commercial types not designated in more detail.

Abbreviations used
NAD — nicotinamide adenine dinucleotide
EDTA — ethylenediamine tetraacetic acid
NBT — nitrotetrazolium blue chloride
TTC — triphenyltetrazolium chloride
ADH — alcohol dehydrogenase

ten plants each in conical flasks fitted with a ground glass seal with two taps. A stream of nitrogen, from which oxygen had been removed in pyrogallol solution, was passed through the flasks. Controls were cultivated in conical flasks without the seal. After anaerobiosis, air was passed through the flasks.

Determination of Ethanol

Ethanol was determined in aqueous plant tissue distillate by means of the enzyme method. The determination is based on ethanol oxidation to acetaldehyde by alcohol dehydrogenase with the aid of NAD, according to the equation

$$C_2H_5OH + NAD \xrightarrow{\text{alcohol dehydrogenase}} CH_3CHO + NADH_2$$

We measured by pipette 0·1 ml 0·024 M NAD, 1 ml standard ethanol solution or aqueous plant tissue distillate and 2·1 ml glycine buffer solution composed of 3 parts 0.1 N NaCl, 7 parts 0·1 N glycine in 0·1 N NaCl, 1 part 0·1 N semicarbazide hydrochloride in 0·1 N NaOH and 0·02 ml enzyme (product of the Boehringer Co., 30 mg ADH/ml) into cells. After incubating for 70 minutes at 25° C we read optical density values at 340 nm on a Spektronom Model 202 spectrophotometer.

Preparation of the Alcohol Dehydrogenase Extract

We homogenised plant tissues in a mixer for 5 minutes with 3 parts b.w. 0·05 M phosphate buffer pH 7·5. We filtered the homogenate over a double layer of gauze and centrifuged for 20 minutes at 10,000 g. We removed the sediment containing cell residues and used the supernatant for activity determinations.

Enzyme Activity Determination

This determination was done by a procedure similar to Racker's method (RACKER 1950). Into cells we measured, by pipette, 100 μmoles ethanol, 0·1 ml 0·024 M NAD solution, 2 ml 0·05 M phosphate buffer pH 7·8 and 0·1 ml enzyme extract. We measured the alcohol dehydrogenase activity with a Spektronom 202 spectrophotometer from the optical density increase at 340 nm against a blank sample containing all the reaction mixture components except ethanol at 25° C. An enzyme amount which under the above conditions causes an optical density increase of 1 unit within 2 minutes was taken as a unit of activity.

Purification of Plant Alcohol Dehydrogenases

We precipitated the alcohol dehydrogenase extract from peas with 2% protamine sulphate solution. After centrifugation (30 minutes at 8 000 g) we precipitated the supernatant with ammonium sulphate from 40 to 60% saturation. We dissolved the precipitated protein in 0·05 M phosphate buffer pH 8·5 and separated it on a column of Sephadex G 150 (4·5 × 45 cm) equilibrated with 0·05 M Tris-HCl buffer solution with 0·001 M EDTA pH 8·3. We joined the active fractions, precipitated with ammonium sulphate to

60% saturation and dissolved the precipitated protein in Tris-HCl buffer pH 8·3.

Electrophoresis of Plant Alcohol Dehydrogenases on Polyacrylamide

We prepared the gel according to a procedure reported by J. B. Davis (Davis 1964). We applied an alcohol dehydrogenase extract prepared in 0·2 M Tris-glycine buffer pH 8·3 (100 μg protein) into a layer of sucrose (32%). Separation of the proteins took place for 20 minutes at 100 V and then for 2 hours at 200 V in 0·2 M Tris-glycine buffer solution with 0·01 M 2-mercaptoethanol. We detected the proteins with Amido Black 10 B. The incubation medium for detecting the activity of alcohol dehydrogenase contained 5 mg NAD, 0·1 ml ethanol, 1 mg phenazinmetasulphate and 1 mg NBT or TTC in 10 ml phosphate buffer 0·05 M, pH 7·8. Incubation lasted 1 hour.

Results and Discussion

Ethanol Formation under Natural and Artificial Anaerobiosis

Under natural anaerobic conditions, *i.e.* in the first days of germination, a number of plants (lentils, lupine, peas, beans, maize, barley, oats, rye, wheat, cucumber, melon, flax, sun flower, rape) accumulate ethanol, which starts to disapear when the testa breaks. For illustration, several examples are shown in Fig. 1. The ethanol concentration also increases under artificial

Fig. 1

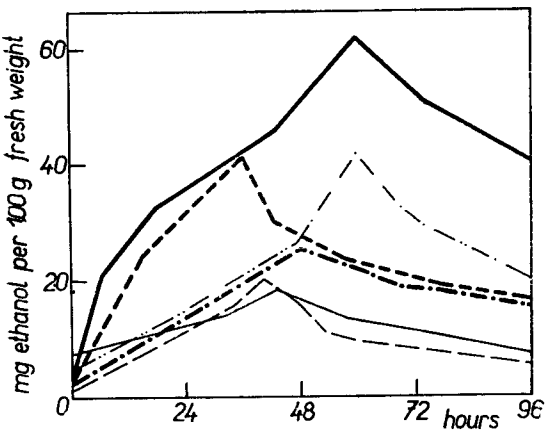

Fig. 2

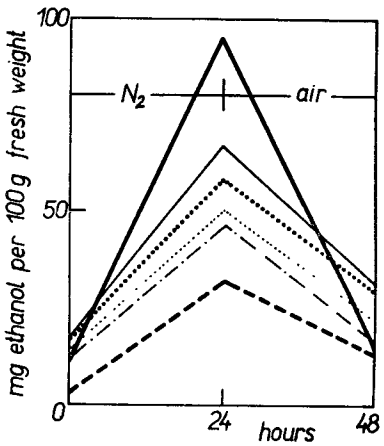

Fig. 1. Ethanol content in germinating plants
abscissa — time of soaking in water (in hours)
ordinate — mg ethanol/100 g fresh weight of plant samples were taken every 6 hours

Fig. 2. Ethanol content in tissues of 14 day old plants after 24 hours of artificial anaerobiosis and 24 hours recovery in air
abscissa — hours
ordinate — mg ethanol/100 fresh weight of plants
Notation see Fig. 3

anaerobic conditions in a nitrgen atmosphere. During 24 hours the ethanol concentration rises several fold (Fig. 2)On returning to the air from the artificial anaerobic conditions, the alcohol content decreases rapidly.

We favour the view that alcohol is degraded by alcohol dehydrogenase, since the alcohol dehydrogenase activity rises in plants under natural anaerobic conditions and under conditions of artificial anaerobiosis (incubation in nitrogen atmosphere), which is evidently due to the increased amount of ethanol accumulated in plants under anaerobiosis. When plants are returned to air from the artificial anaerobic conditions, the enzyme activity decreases in the reserve organs of 1 to 13 day-old pea, maize and sun flower plants, while it slightly rises in the vegetative organs on exposure to air. The latter phenomenon may be due to transport of ethanol from the reserve organs to vegetative ones (LEBLOVÁ *et al.* 1969).

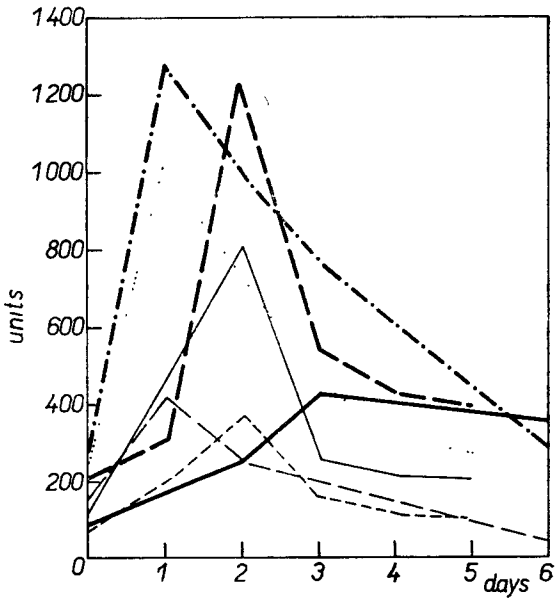

Fig. 3. Specific activity of alcohol dehydrogenase prepared from plants of different ages
abscissa — age of plants in days
ordinate — specific activity in units

Notation identical for Figs. 1, 2, 3

peas
wheat
rape
lentils
oats
rye
barley
maize
flax
sunflower
beans

In plants cultivated in light, the specific activity of alcohol dehydrogenase is greater than that of plants grown in the dark (Table 1).

Purification of Alcohol Dehydrogenases

Considerably less is known of plant alcohol dehydrogenases compared to animal alcohol dehydrogenases (PIETRUSZKO and THORELL 1969), those from yeast (HAIS and VELICK 1954) or *e.g.* from *Drosophila* (SOFER and URSPRUNG 1968). This is apparently due to the high degree of instability of the enzyme on purification (COSSINS *et al.* 1968; DUFFUS 1968; HAGEMAN and FLESHER 1960; PATTEE and SWAISGOOD 1968). Some of the above authors succeeded in preparing the animal and yeast enzyme in crystalline form, while no one has so far succedded in preparing plant alcohol dehydrogenase in this state.

TABLE 1

SPECIFIC ACTIVITY OF ALCOHOL DEHYDROGENASE (expressed in units) prepared from pea, maize and sunflower plants of different ages, cultivated in light as well as in the dark under aerobic and anaerobic conditions*)

Plant	Manner of cultivation	Time of germination						Age of plants (days)								
		1	2			3		6			9			13		
			K	N_2	V			K	N_2	V	K	N_2	V	K	N_2	V
Peas	in light	143	188	232	200	445	R. o.	380	420	400	350	485	340	271	350	197
							V. o.	338	350	618	209	343	410	108	152	180
	in dark	134	178	212	180	406	R. o.	306	350	274	191	246	195	144	248	132
							V. o.	295	330	596	85	127	205	61	166	216
Maize	in light	1240	930	1030	830	770	R. o.	445	614	396	130	189	69	63	146	60
							V. o.	280	375	436	100	177	280	57	117	171
	in dark	1100	780	903	805	680	R. o.	207	314	122	109	147	47	52	135	59
							V. o.	150	226	288	69	112	238	39	58	100
Sun flower	in light	396	189	243	173	172	R. o.	15	27	17	13,5	46	10	8	19	9
							V. o.	13	27	38	12	25	40	6	17	60
	in dark	210	164	190	163	120	R. o.	12	21	14	7	28	9	6	18	5
							V. o.	8	13	31	6	11	25	2	15	30

K — control
N_2 — cultivated for 24 hours in nitrogen
V — cultivated for 24 hours in nitrogen and then 24hours in air
R. o. — reserve organs
V. o. — vegetative organs

*) One representative experiment was selected in every case out of a series of five

TABLE 2
PURIFICATION OF ALCOHOL DEHYDROGENASE from peas

Fraction	Total protein mg	Total activity units $\times 10^6$	Specific activity units/mg protein
Extract	4 500	1·89	420
Supernatant obtained after precipitating extract with protamine sulphate	2 880	2·04	740
Supernatant obtained after precipitating with ammonium sulphate to 40% saturation	990	1·62	1 640
Solution obtained by dissolving the protein salted out with ammonium sulphate from 40 to 60% saturation	432	1·08	2 500
Solution obtained by joining the fractions after gel filtration	195	0·176	907
Solution obtained by dissolving the protein precipitated by ammonium sulphate to 60% saturation from the combined fractions after gel filtration	119	0·022	196

Table 2 shows the results of purification of the enzyme from two-day old pea plants. On precipitation of the enzyme extract with protamine sulphate the activity increased twice, on precipitation with ammonium sulphate from 40% to 60% saturation six times, while gel filtration resulted in a specific activity decrease.

In the case of lentils the fraction obtained by precipitating the extract with ammonium sulphate from 40% to 60% saturation had a specific activity three times higher than that of the original extract, in the case of rape six times; with cereals activity was nearly lost on saturation with ammonium sulphate.

Properties of Plant Alcohol Dehydrogenases

Pea, maize and sunflower alcohol dehydrogenase is contained in the soluble fraction not sedimenting at 120 000 g.

From the relationship of reaction rate and substrate concentration the Michaelis constant was estimated and found to be $1{\cdot}75 \times 10^{-5}$ M in the case of the enzyme prepared from peas, 1.50×10^{-5} M with that from maize and $1{\cdot}21 \times 10^{-5}$ M with that from sun flower.

The optimum pH value for plant alcohol dehydrogenases is in the slightly alkaline region (Table 3).

It was found with the aid of inhibitors reacting with —SH groups, that these groups are contained in plant alcohol dehydrogenases and that they are essential for this enzyme. It was similarly established by reacting the enzyme with metal-binding substances, that alcohol dehydrogenase is a metaloprotein (COSSINS and TURNER 1962, APP and MEISS 1958, SUZUKI 1966, COSSINS *et al.* 1968).

TABLE 3

pH OPTIMUM of plant alcohol dehydrogenases

Plant type	Optimum pH
Lentils	7·5
Peas	8·0
Rye	8·4
Wheat	8·5
Barley	8·7
Oats	8·7
Sunflower	8·8

We have therefore also investigated the influence of —SH containing substances, *i.e.* mercaptoethanol and L-cystein (10^{-2} M solution) on activation of the enzyme, finding that mercaptoethanol activates the purified enzyme only (the enzyme obtained by salting out fractions after gel filtration). No such activating influence was found on the enzyme extract prepared from peas, maize and sunflower and the enzyme precipitated from this extract with ammonium sulphate. Activation by L-cystein was indistinct. Inhibition of the activity of enzyme extracts, expressed in percentages, is summarized in Table 4. (Inhibition by Zn^{2+} and Cu^{2+} ions was measured in Tris-HCl

TABLE 4

INFLUENCE OF EDTA, p-choloromercuric benzoate, iodoacetamide, copper, zinc and boron ions on the alcohol dehydrogenase activity

Inhibitor	Concentration	Inhibition percentage					
		Peas	Beans	Wheat	Maize	Sun flower	Rape
P-chloromercuric benzoate	10^{-5} M	100	100	100	100	100	100
Iodoacetamide	10^{-3} M	8	7	16	14	10	17
EDTA	10^{-2} M	31	40	40	44	38	36
$B_4O_7^{2-}$	10^{-2} M	83	90	84	89	87	78
Zn^{2+}	10^{-3} M	50	57	16	19	39	30
Cu^{2+}	10^{-3} M	98	93	97	99	91	86
Hg^{2+}	10^{-3} M	—	90	100	—	100	100

buffer solution pH 7·8. In the other experiments a phosphate buffer of pH 7·8 was used).

Isoenzymes of Plant Alcohol Dehydrogenases

On separating the enzyme extract of germinating seeds by electrophoresis on polyacrylamide we determined the isoenzyme composition of plant alcohol dehydrogenase. Alcohol dehydrogenase from maize contains two isoenzyme groups, of which the one which migrates to the anode more rapidly contains one isoenzyme, the more slowly migrating group is composed of four isoenzymes. In the case of alcohol dehydrogenase from barley we similarly succeeded in proving the existence of five isoenzymes, in the case of wheat and oats six and in the case of peas and sun flower three isoenzymes in each case.

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SYLVA LEBLOVÁ, ILONA ZIMÁKOVÁ, JANA BARTHOVÁ, DANA EHLICHOVÁ, Katedra biochemie Přírodovědecké fakulty Karlovy university Praha: **Rostlinné alkoholdehydrogenasy.**— Biol. Plant. **12** : 33—42, 1970.

Zjistily jsme, že u celé řady rostlin (čočky, lupiny, fazolu, pelušky, ječmene, ovsa, žita, pšenice, okurky, melounu, lnu, slunečnice, řepky olejky) se za přirozené anaerobiosy tvoří větší nebo menší množství ethanolu a že všechny tyto rostliny reagují i v dalším růstovém údobí na anaerobiosu tvorbou ethanolu. Ethanol po prasknutí testy u klíčících rostlin nebo přenesení rostlin z anaerobní atmosféry na vzduch mizí.

Rostliny obsahují alkoholdehydrogenasy, jejichž aktivity je závislá na koncentraci ethanolu v pletivech: dosahuje maxima během přirozené anaerobiosy a v dalším růstu se zvýší při přechovávání rostlin v atmosféře dusíku.

Alkoholdehydrogenasy studovaných rostlin jsou lokalisované v rozpustné buněčné frakci nesedimentující při 120 000 g, mají pH optimum v mírně alkalické oblasti a Michaelisovy konstanty jsou řádově stejné (10^{-5} M). Inhibice ionty Zn^{2+}, Cu^{2+}, Hg^{2+}, $B_4O_7^{2-}$, p-chlormerkuti-benzoátem, jodacetátem, EDTA a fenantrolinem jsou obdobné a svědčí o přítomnosti —SH skupin. Specifická aktivita preparátu alkoholdehydrogenasy je vyšší u rostlin pěstovaných na světle než ve tmě.

Srážení síranem amonným dovolí zvýšit specifickou aktivitu rostlinných alkoholdehydrogenas maximálně o jeden řád, u obilovin se u tohoto stupně čištění aktivita ztrácí.

Elektroforesou na polyakrylamidu jsme zjistily isoenzymové složení ADH: Např. enzym z hrachu a slunečnice obsahuje 3, pšenice a oves 6 a kukuřice a ječmen 5 isoenzymů.