ALCOHOL DEHYDROGENASE ACTIVITY IN THE COTYLEDONS OF PEAS DURING MATURATION AND GERMINATION

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SUMMARY

A quantitative biochemical and histochemical study of the alcohol dehydrogenase activity of pea cotyledons indicated that the enzyme activity decreased about 40% during the first seven days of germination. The enzyme is formed during seed development and is reactivated by hydration only during germination.

During homogenization of cotyledons of peas germinated for several days, substances are liberated which inactivate the enzyme. These substances – very probably long-chain fatty acids – are absent in the intact cotyledons and are also absent in the cotyledons and homogenates of seeds germinated for one day. To ascertain the alcohol dehydrogenase activity of cotyledons of peas germinated for several days, it is necessary to add bovine serum albumin to the extraction medium or to avoid homogenization of the tissue by using a histochemical method.

1. INTRODUCTION

Germinating pea seeds always experience a period of partial anaerobiosis caused by the poor permeability for O_2 of the intact seed coat (FERNANDES 1923; FRIETINGER 1927; SPRAGG & YEMM 1959; KOLLÖFFEL 1967). This period of natural anaerobiosis is accompanied by an accumulation of lactic acid (COSSINS 1964) and ethanol (COSSINS & TURNER 1959; 1962; 1963) and ceases when the radicle ruptures the seed coat. The accumulation of ethanol largely depends on the conditions during germination (DOIREAU & DUPÉRON 1966; LEBLOVÁ *et al.* 1969). When the seedling develops under aerobic conditions, lactic acid and ethanol are metabolized as shown by experiments with ^{14}C-labeled lactate and ethanol (COSSINS 1962; COSSINS & BEEVERS 1963; COSSINS & TURNER 1963; CAMERON & COSSINS 1967).

The enzymes involved in the anaerobic respiration have been studied frequently. There is relatively much known about the physical and catalytic properties of alcohol dehydrogenase (ADH) from pea cotyledons (SUZUKI 1966; ERIKSSON 1968; COSSINS *et al.* 1968). The activity decreases a few days after the onset of germination (VIRTANEN 1944; DAVISON 1949; GOKSÖYR *et al.* 1953; COSSINS & TURNER 1962; COSSINS *et al.* 1968; KOLLÖFFEL 1968) but remains high when the seeds were allowed to germinate under anaerobic conditions (MAFFEI FACCIOLI 1959; KOLLÖFFEL 1968).

The histochemical localisation of ADH activity in germinating pea cotyledons has recently been described (KOLLÖFFEL 1969). It was found that the ADH activity of 7-day-old cotyledons was only somewhat lower than that of 1-day-

old cotyledons in contrast with earlier results obtained with biochemical assays (KOLLÖFFEL 1968). In the present investigation, these studies have been extended. It will be shown that the extracts from cotyledons of seeds germinated for several days contain inhibitors of ADH, which are absent in extracts from newly germinated seeds.

2. MATERIALS AND METHODS

2.1. Germination conditions

Air-dry seeds of *Pisum sativum* L. cv. "*Rondo*" were soaked in aerated tap water for 20–22 hrs at 23° in darkness and next transferred to moist filter paper in large Petri dishes for further germination. The cotyledons of peas germinated for e.g. 7 days will be mentioned as "7-day-old" cotyledons.

2.2. Preparation of the sections and conditions of incubation

Freshly cut freehand sections of the cotyledons were thoroughly washed with phosphate buffer and incubated in a medium containing: phosphate buffer (0.05 M, pH 6.4), NAD (1.35 mM), ethanol (0.4 M) and Nitro-BT (nitro-blue tetrazolium chloride; The British Drug Houses Ltd.). After 30 min of incubation at 25° the reaction was stopped by washing the sections thoroughly with 0.1 N HCl. The unspecific reduction of Nitro-BT by endogenous substrates was followed by incubating sections in a medium without ethanol. Details of this procedure have been described elsewhere (KOLLÖFFEL 1969).

2.3. Extraction of the formazan of Nitro-BT

A few ml of a mixture of N,N-dimethylformamide and 0.1 N HCl (1:1) were added to the stained sections and this was carefully heated to 80°. This temperature may not be exceeded because otherwise the sections are destroyed. After a few minutes the extract was collected and the procedure was repeated twice. The combined extracts were centrifuged to remove cell debris. The amount of formazan was determined by comparing the optical density of the centrifuged extract at 540 nm with that of a standard solution of chemically reduced Nitro-BT.

Because of its high affinity for cellular structures Nitro-BT was not completely washed from the sections after incubation and, as it is easily reduced unspecifically at alkaline pH, it was necessary to perform all operations at an acid pH.

After incubation, the sections were dried for about 20 hrs at 60° and next weighed.

ADH activity was expressed as mg formazan produced per g tissue (dry weight) per 30 min. The activity was always corrected for a slight unspecific reduction of Nitro-BT occurring in the control sections.

Although the formazan was extracted only partially from the sections, the method described is yet a direct measure of the alcohol dehydrogenase activity because it recorded a linear response with respect to the time of incubation and also to the amount of tissue – and hence enzyme – present.

2.4. Preparation of extracts from swollen and air-dry cotyledons

Prechilled cotyledons (40) were ground in a mortar with 10 g sand and 10 ml medium containing: 0.4 M sucrose, 0.05 M phosphate buffer (pH 7.2) and 2% bovine serum albumine (BSA). The resulting slurry was squeezed through double cheesecloth and the volume was made up to 35 ml with grinding medium. The homogenate was then centrifuged at 1500 × g for 10 min. The supernatant fraction was recentrifuged, now at 20,000 × g for 15 min, and the supernatant fraction thus obtained was used as the source of alcohol dehydrogenase. Air-dry cotyledons were first pulverized with a "multimix" (BRAUN A.G., Frankfurt a. M.). An equal weight of tap water was added to the powder, and this was next allowed to imbibe for 2 hrs at 0°–5°. Thereafter the imbibed powder was treated in the same way as preparations from fresh cotyledons.

2.5. Assay of alcohol dehydrogenase activity (Alcohol:NAD oxidoreductase E. C. 1.1.1.1)

The reaction mixture contained: 0.05 M phosphate buffer, 0.01 M acetaldehyde, 0.225 mM NADH and 0.1 ml enzyme preparation in a total volume of 3.0 ml with a pH of 7.2. About 15 sec after the addition of acetaldehyde, the decrease in absorbance at 340 nm was followed at 25° in a Unicam S.P. 800 recording spectrophotometer against a blank containing enzyme, buffer and NADH. The reaction proceeded linearly with time for 1 to 3 min. Enzyme activity was calculated from the initial reaction velocities of at least three different dilutions of one extract. A molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1} was used.

Acetaldehyde was distilled before use and diluted with water. A 0.2 M solution was stored at –8° for several days.

3. RESULTS AND DISCUSSION

Extracts from 1-day-old cotyledons showed a sizable ADH activity but in those from 7-day-old cotyledons the activity was very low (*table 1*, left column).

Table 1. Effect of bovine serum albumin (BSA) on the alcohol dehydrogenase activity of extracts from cotyledons of germinating peas. Extracts were prepared in the absence (–BSA) or presence (+BSA) of BSA (2%). Enzyme activity expressed as μmoles of NADH oxidized per cotyledon per min.

germination period in days	–BSA	+BSA
0	40	39
1	46	58
3	35	45
5	12	34
7	4	25

Table 2. Inactivation of the alcohol dehydrogenase activity of an extract from 1-day-old cotyledons by an extract from 7-day-old cotyledons. The extracts were mixed (1:1) and kept in ice for the appropriate time. Enzyme activity expressed as μmoles NADH oxidized per cotyledon per min.

treatment	time after mixing min	activity	% of calculated activity
1-day-old		46	
7-day-old		4	
mixture (1:1)	0	25	100
	60	14	56
	120	10	40
	180	8	32
	300	7	28
+2% BSA	300	24	96
+0.01 M glutathione	300	8	32

When these two extracts were mixed the resulting ADH activity was much lower than that which could be expected from the added individual values (*table 2*). The depression of the ADH activity of the combined extracts increased with time. It could be prevented completely, however, by adding bovine serum albumin (BSA) to the mixture of the two extracts (*table 1*). Glutathione had only little effect. Inactivation of the ADH activity did not occur when two extracts from 1-day-old cotyledons were mixed. When sections of 1-day-old cotyledons were put in an extract from 7-day-old cotyledons for about 1 hr at 4°, the ADH activity of these sections was much lower than that of controls put in buffer or in an extract from 1-day-old cotyledons.

It is clear that extracts from older cotyledons contain substances which are able to inactivate ADH. Now the question arose whether the low ADH activity of extracts from older cotyledons resulted from some kind of inactivation during preparation of the extracts. Therefore BSA was added to the extraction medium (*table 1*, right column). The effect of BSA largely depended on the germination period of the cotyledons used. It was without effect on the activity of extracts from air-dry cotyledons but that from 7-day-old cotyledons was greatly enhanced.

A similar artifact of isolation has been described by ZEEVAART *et al.* (1968). They showed that the decrease in the activity of some mitochondrial characteristics in pea cotyledons during germination was not due to changes in the mitochondria themselves, but to an increased concentration of free fatty acids on the mitochondria. These fatty acids were formed during homogenization of the tissue and were bound by BSA. As it is also known (ERIKSSON 1968) that ADH from pea cotyledons is inhibited by fatty acids it was investigated whether the low ADH activity from 7-day-old cotyledons is caused by these substances.

A crude uncentrifuged homogenate was kept in ice for 1 hr. The lipid material was extracted from this with a chloroform-methanol mixture by the method of

Table 3. The alcohol dehydrogenase activity in sections of germinating pea cotyledons assayed with nitro-blue tetrazolium and expressed as mg formazan produced per g tissue (dry weight) per 30 min.

germination period in days	mg formazan per g per 30 min
1	0.73
3	0.59
5	0.53
7	0.45

BLIGH & DYER (1959). The extracted lipids were fractionated by thin-layer chromatography (TLC) on plates of Kieselgel HF (E. Merck, Darmstadt). The plates were developed with petroleum ether (b.p. 40–60°)-ether-formic acid (60:40:1.5, by vol.) and the spots were visualized by exposure to iodine vapour. A comparison of a TLC of an extract from 1-day-old cotyledons with that of 7-day-old cotyledons showed that fatty acids and monoglycerides were present almost exclusively in the extract of 7-day-old cotyledons. These substances were absent, however, when pea cotyledons themselves were homogenized in methanol. The fatty acids were analyzed by gasliquid chromatography after they had been eluted from the gel and methylated with methanol-sulfuric acid (95:5, by weight). The main component of the mixture was linoleic acid (about 60%) whereas smaller amounts of oleic acid (about 10%) and palmitic acid (about 10%) and much smaller amounts of stearic acid, linolenic acid and short-chain fatty acids were present. These results are in good agreement with those reported earlier (ERIKSSON 1967; ZEEVAART *et al.* 1968). The inhibitory action of linoleic acid and oleic on the ADH activity of an extract from 1-day-old cotyledons is shown in *table 4*. Methanol only – the solvent – inhibited the reaction only weakly.

These results thus clearly indicate that during homogenization of 7-day-old cotyledons fatty acids are liberated which are absent in intact cotyledons and also in 1-day-old cotyledons and their homogenates. As these fatty acids are

Table 4. Effect of oleic acid and linoleic acid on the alcohol dehydrogenase activity of an extract of 1-day-old cotyledons. Ten µl of a solution of oleic acid (0.3 M) or linoleic acid (0.3 M) in methanol were added to 10 ml of a strongly diluted enzyme preparation. The mixture was stirred thoroughly in nitrogen atmosphere at 4°. A sample was pipetted into a cell and 0.15 ml NADH and 0.15 ml acetaldehyde were added. Enzyme activity expressed as µmoles NADH oxidized per cotyledon per min.

addition to the	preincubation time in min		
enzyme preparation	0	15	30
none	56	57	54
methanol	44	45	42
+ oleic acid	42	15	4
+ linoleic acid	42	7	1

able to inactivate ADH it is probable that they are involved in the *in vitro* inactivation of ADH from 7-day-old cotyledons. It is generally known that fatty acids are bound by BSA. The present experiments do not exclude the possibility, however, that other substances are also involved in the inactivation of ADH.

When the ADH activity of the cotyledons was determined histochemically it appeared that sections of 7-day-old cotyledons showed an activity which was only about 40% lower than that of sections of 1-day-old ones. These results are consistent with those of extracts to which BSA was added. To ascertain the ADH activity of older cotyledons, it is thus necessary to add BSA to the extraction medium or to avoid homogenization of the tissue by using a histochemical method. The results of both methods indicate anyway that the ADH activity of pea cotyledons decreased about 40% over a germination period of 7 days.

Extracts from air-dry cotyledons showed a sizable ADH activity which implies that the enzyme must have been synthesized during the development of the seed. Experiments with maturing pea seeds indicated that the highest ADH activity was reached at a relative water content of the cotyledons of about 47% (water content as percentage of the fresh weight). The decrease of the relative water content from 50 to 13% (air-dry, mature seeds) during maturation was accompanied by a decrease of about 15% of the ADH activity. So it is clear that the enzyme is reversibly inactivated during maturation of the seed and reactivated by hydration only during germination. In this respect it resembles the mitochondrial malate and succinate dehydrogenase activity (KOLLÖFFEL 1970; KOLLÖFFEL & SLUYS 1970).

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