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The Effect of Metals on Isolated Pea Pyruvate Decarboxylase

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Abstract. Pyruvate decarboxylase (PDC) was prepared from four-day-old pea seedlings by a procedure which involves extraction of plant material with a phosphate buffer, fractionation of the extract with ammonium sulphate, desalting by dialysis or gel filtration on Sephadex G-25 column, chromatography on DEAE cellulose and filtration on Sepharose 4B. The PDC preparation activity 10 000 U g^{-1} protein was about 600 fold higher than that of the sodium phosphate buffer extract. According to the enzyme behaviour during the gel filtration on different carriers the molecular mass of pea PDS was estimated at about 10^6. Magnesium ions and thiamine pyrophosphate were found to be coenzymes of PDC. Cofactors can be removed by 48 h dialysis followed by chromatography on DEAE cellulose. Apoenzym is activated optimally with the concentration of cofactors of 0.002 M. Magnesium ions can be replaced in their activation function by ions of Fe^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+} and Mn^{2+}. Another ions, *i.e.* Ba^{2+}, Ca^{2+}, Cd^{2+}, Hg^{2+} and Cu^{2+} are inactive. Assumption about the relation between the ion diameter and degree of activation is formulated.

Anaerobic glucose degradation during the germination of seeds was investigated in one of our previous papers (LEBLOVÁ *et al.* 1974). We found that ethanol and lactate are formed during this process. The first enzyme which is involved in pyruvate metabolism during the alcoholic fermentation is pyruvate decarboxylase (PDC) catalyzing formation of acetaldehyde. This enzyme has not so far been extensively studied in plant material (KENWORTHY and DAVIES 1976). We present here a procedure for preparation of pea pyruvate dehydrogenase of high activity. Results of our experiments showed possibility of replacing magnesium bound in PDC protein with some other metals. The finding that PDC activity is significantly changed by cations present in the medium is a practical contribution.

MATERIAL AND METHODS

Cultivation of Plants

Four-day-old germinating seeds of pea (*Pisum sativum* L. cv. Pyram) were used. Fifty seeds were germinated at 18 °C on a filter paper in a dish (15 cm in diameter) containing 25 ml of water.

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TABLE 1
Isolation of pea pyruvate decarboxylase (PDC). (Data presented in the upper two lines are calculated for 100 g of germinating seeds)

Source of PDC	Protein [g]	Activity (U)	Activity (U g^{-1} protein)	Purified
Extract	3.45	86	25	1×
Ammonium sulphate fraction	0.175	43	200	8×
Desalted ammonium sulphate fraction	0.11	31.9	290	12×
Active fraction after DEAE cellulose	0.0024	7.2	3 000	120×
Active fraction after Sepharose 4B	0.0004	7.0	15 000	600×

Estimation of PDC Activity

Two procedures were used: The first spectrophotometric method is based on reduction of originating acetaldehyde by alcoholdehydrogenase with NADH as coenzyme. The decrease in absorbance is measured at 366 nm. The following components were pipetted into a test tube: 0.2 ml of TRIS-acetate buffer, pH 6.0, 0.25 ml of water, 0.1 ml of 0.01 M thiamine pyrophosphate (TPP), 0.05 ml of 3 mM NADH, 0.1 ml of 0.01 M $MgCl_2$, 0.15 ml of 0.2 M pyruvate and 0.05 ml of alcohol dehydrogenase (activity 98700 U g^{-1}). Reaction was started by addition of 0.1 ml of PDC (KENWORTHY and DAVIES 1976).

The second fluorometric method follows the formation of acetaldehyde-dimedone complex. The incubation mixture consists of 0.2 ml of 0.2 M TRIS-acetate buffer, pH 6.0, 0.2 ml of 0.2 M pyruvate, 0.1 ml of 0.01 M TPP, 0.1 ml of 0.01 M $MgCl_2$ and 0.1 ml of PDC diluted to a concentration which liberates about 0.0005 M of acetaldehyde. After 10 min of incubation at 37 °C 1 ml of dimedone reagent was added and the mixture was heated for 6 min in a boiling water bath (LEBLOVÁ and VALÍK 1980). Then the fluorescence was measured.

The unit of the activity is such an amount of PDC which catalyzes formation of 1 mmol of acetaldehyde in 1 min.

Isolation of Pyruvate Decarboxylase

Four days old pea seedlings (50 g) were homogenized in 50 ml of 0.2 M sodium phosphate buffer, pH 6.0. The homogenate was centrifuged at 10000 *g* for 10 min. The pH of the supernatant was adjusted with sodium hydroxide to 6.0 and PDC was precipitated by addition of ammonium sulphate to 10–30% saturation. Precipitate was dissolved in 0.2 M sodium phosphate buffer, pH 6.0, containing 0.0005 M of mercaptoethanol. The solution was desalted either by dialysis or on Sephadex G-25. PDC was further purified by chromatography on a DEAE cellulose column (2.5×40 mm) which was eluted with TRIS-acetate buffer (pH 6.0) gradient from 0.02 M to 0.6 M. Fractions of 15 ml were collected at 20 min intervals. For gel filtration

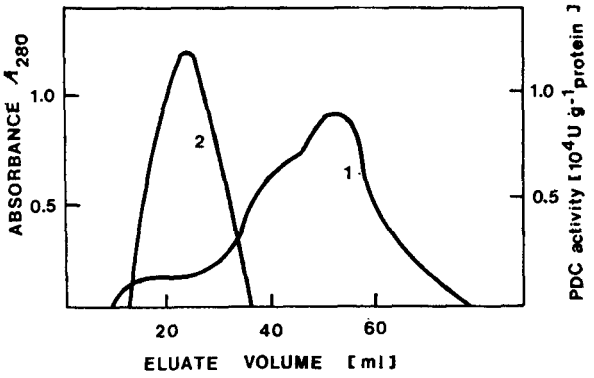

Fig. 1. Sepharose 4B gel filtration of pyruvate decarboxylase (PDC) isolated from four-day-old pea seedlings. Abscissa: volume of eluate in ml; ordinate: absorbance at 280 nm (left) and PDC activity in U g^{-1} protein (right). Curves 1 and 2 correspond to protein and PDC activity, respectively.

fractions precipitated with ammonium sulphate were dissolved as described above, applied on Sephadex G-200 column (3 × 70 cm) and eluted with 0.02 M TRIS-acetate buffer, pH 6.0. Three ml fractions were collected at 20 min intervals. Fractionation on Sepharose 4B proceeded on a 1 × 40 cm column operating at 4 °C. Concentrated active fractions obtained after DEAE cellulose chromatography were applied on this column which was again eluted with 0.02 M TRIS-acetate buffer. One ml fractions were collected at 20 min intervals at 4 °C.

Estimation of Proteins

Proteins were estimated by the method of LOWRY *et al.* (1951) using serum albumin as a standard.

TABLE 2

Removal of cofactors from pea pyruvate decarboxylase (PDC). (Activity of PDC with 0.002 M TPP and Mg^{2+} is considered as 100 %)

Isolation step	TPP [M]	Mg^{2+} [M]	Activity [%]
Extract	0	0	100
	0.002	0	100
	0.002	0.002	100
Ammonium sulphate fraction	0	0	100
	0.002	0	100
	0.002	0.002	100
Ammonium sulphate fraction dialyzed 24 h	0	0	75
	0.002	0	85
	0.002	0.002	100
Ammonium sulphate fraction dialyzed 48 h	0	0	60
	0.002	0	65
	0.002	0.002	100
Ammonium sulphate fraction dialyzed 48 h and then chromatographed on DEAE cellulose	0	0	0
	0.002	0	20
	0.002	0.002	100

TABLE 3
The effect of metals on pea pyruvate decarboxylase (PDC) and its apoenzyme

Cation	Cation concentration 10^{-3} M	Mg^{2+} concentration 10^{-3} M	Activity [%]
Mg^{2+}	0	0	0
	0	2	100
Co^{2+}	2	0	115
	2	2	135
Ni^{2+}	2	0	106
	2	2	114
Zn^{2+}	2	0	105
	2	2	110
Fe^{+2}	2	0	100
	2	2	105
Mn_2^{+}	2	0	75
	2	2	90
Ba^{2+}	2	0	0
	2	2	100
Ca^{2+}	2	0	−10
	2	2	35
Cr^{3+}	2	0	−2
	2	2	45
Fe^{3+}	2	0	−2
	2	2	40
Cd^{2+}	2	0	−15
	2	2	−4
Hg^{2+}	2	0	−12
	2	2	−10
Cu^{2+}	2	0	−16
	2	2	−14

RESULTS AND DISCUSSION

Procedure for the purification of pea PDC is shown in Table 1. It involves precipitation of protein with ammonium sulphate to 15—30% saturation, desalting of the active fraction by dialysis or by filtration on Sephadex G-25. The desalted fraction is then applied on the DEAE cellulose column and collected active fractions are precipitated with ammonium sulphate added to 0—50% of saturation. The precipitate dissolved in sodium phosphate buffer is applied on a Sepharose 4 B column (Fig. 1) and eluted. Using this procedure the activity of the final preparation was 600 fold higher than that of the original sodium acetate buffer extract. Gel filtration did not

increase enzymatic activity. PDC was eluted together with the majority of proteins at the front from the columns of Sephadex G 200, CM cellulose as well as phosphocellulose. This indicates that PDC has a high molecular mass. DAVIS (1976) estimated the molecular mass of the PDC so far isolated from wheat germ as 570 000. The high molecular mass of PDC and sigmoid dependence of pea enzyme on pyruvate concentration (LEBLOVÁ and VALÍK 1980) indicate that this enzyme has a regulatory function in plant cells. According to the results summarized in Table 2 pea PDC is functional together with magnesium ions and TPP. Apoenzyme can be prepared by 48 h dialysis which is followed by chromatography on DEAE cellulose. It can be activated by 0.002 M concentrations of coenzymes.

Table 3 shows that apoenzyme can be reactivated not only by magnesium ions but also by ions of some other metals.

Bivalent ions of iron, nickel, cobalt, zinc and manganese are active, which were tested as chlorides in incubation medium. It seems that reactivation is dependent on (1) the electric charge (bivalent ions are activating, trivalent ions inhibiting) and (2) size of ions. When bivalent ions are arranged in a row according to their ion diameters and compared with changes of pea PDC activities in the presence of a single ion as well as together with Mg^{2+} one finds that the activity is increasing in order magnesium, iron, nickel and cobalt (diameter from 0.65 to 0.77). With Zn^{2+} (diameter 0.83) and Mn^{2+} (diameter 0.91) the activity decreases. Ca^{2+} and Cd^{2+} (diameter 0.99 and 1.03, respectively) are not active alone: One can expect that their size enables them to penetrate to the magnesium binding site and that they affect the binding of magnesium. On the other hand Ba^{2+} ions (ionic diameter 1.35) have no effect on the activity of pea PDC. Evidently they are so voluminous that they do not penetrate to the site of protein-magnesium interaction and do not compete with the magnesium ion, for the binding site of pea PDC. The process of activation is also affected by the ion redox state as is evident in the case of iron: Its bivalent cation acts as activator while the trivalent one has an inhibiting effect. Some bivalent ions as Cd^{2+}, Cu^{2+} and Hg^{2+} also act as inhibitors. The last two ions probably irreversibly inhibit the activity by interaction with sulphydryl or carboxyl groups of protein. It seems that pea PDS is an enzyme the activity of which can be significantly affected by endogenous ions including ions derived from soil and synthetic fertilizers. Attention should be paid to this point because PDC represents one of the most important enzymes the catalyzing effect of which makes it possible to supply seeds with adenosine triphosphate originating at a substrate level.

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