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ISOLATION AND PROPERTIES OF LACTATE DEHYDROGENASE FROM GERMINATING PEA PLANTS

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Abstract—Lactate dehydrogenase (LDH) was isolated from pea seedlings by means of protamine sulphate and $(NH_4)_2SO_4$ fractionation and chromatography on DEAE-cellulose and Sephadex G-150. The enzyme had a MW of *ca* 145500. The kinetic properties studied were the lactate oxidation pH optimum (9·1) and the pyruvate reduction pH optimum (7·1). K_m values were determined for four natural substrates (lactate, pyruvate, NAD^+ and NADH) and for other acids (glycollate, α-ketoglutarate and glyoxylate). The K_i value was determined for *p*-chloromercuribenzoate (PCMB) which is a noncompetitive inhibitor of LDH from pea plants, and the course of irreversible inhibition of the enzyme by iodoacetamide (IA) and *n*-ethylmaleimide (NEMI) was studied. Preincubation of LDH with the coenzyme protects against PCMB inhibition, indicating the important role of the sulfhydryl group in the active site.

INTRODUCTION

The period of natural anaerobiosis, which all plants experience during seed germination, is accompanied by marked alterations in ethanol and lactate concentration [1–4]. Both products originate under anaerobic conditions from pyruvate, the first-named under catalysis by pyruvate decarboxylase and alcohol dehydrogenase, the latter by lactate dehydrogenase [2,5]. In germinating seeds LDH probably catalyses formation as well as degradation of lactate. As compared with LDH from animal sources little attention has been paid so far to studying the enzyme in plants. LDH has been isolated from soybean cotyledons [5], and its properties studied in potato tubers [6,7] and in *Hevea brasiliensis* latex [8,9]. In earlier studies [10,11] we investigated the properties and function of LDH in germinating soybeans.

In the present work we describe a procedure for purifying the enzyme from germinating pea plants, and some kinetic and structural properties of the partially purified enzyme with respect to the function of LDH in anaerobic plant metabolism.

RESULTS AND DISCUSSION

Isolation of LDH

During the germination of pea plants, the sp. act. of LDH rises at first reaching a maximum after 48 hr—*ca* ten-fold in comparison to the dry seed—and then decreases. We used seedlings aged 48 hr as a source of the enzyme. The enzyme was extracted with a buffer containing EDTA to bind heavy metals, 2-mercaptoethanol to protect the –SH groups of the enzyme and Triton X-100. The extract was purified by precipitation with protamine sulphate and ammonium sulphate. The protein fraction with LDH activity precipitated at ammonium sulphate saturation levels of 30–40%. The most efficient step in the entire isolation procedure was chromatography on DEAE-cellulose. In this step, activity of the preparation increased *ca* eight-fold. The final step in the isolation procedure was gel filtration on Sephadex G-150. The LDH preparation obtained had a sp. act. *ca* 190 times higher as compared to the crude extract; when stored at 4° the preparation kept its activity for at least one week.

Properties of LDH from germinating pea plants

pH optimum. There was a sharp maximum at pH 9·1 in the direction of lactate oxidation and at pH 7·1 in the direction of pyruvate reduction. In this respect the pea enzyme behaves similarly to the enzyme isolated from animal sources or plants [5,10].

Substrate concentration. K_m values for lactate, pyruvate, NAD^+ and NADH were 5×10^{-2}, $3{\cdot}3 \times 10^{-4}$, 1×10^{-3} and $2{\cdot}8 \times 10^{-5}$ M respectively, determined by the Lineweaver–Burk method. In this respect, the pea enzyme does not differ significantly from LDH obtained from animal sources [12].

Substrate specificity. LDH of pea plants catalysed the oxidation of glycollate and reduction of α-ketoglutarate and glyoxylate in the presence of NAD^+ or NADH as coenzymes. The K_m values for lactate, glycollate, pyruvate, α-ketoglutarate and glyoxylate were 5×10^{-2}, $7{\cdot}2 \times 10^{-2}$, $3{\cdot}3 \times 10^{-4}$, $3{\cdot}4 \times 10^{-3}$ and $1{\cdot}6 \times 10^{-2}$ M respectively. LDH from soybeans [5,11] also had a wider specificity. The results on substrate specificity of potato enzyme differ according to authors. Whereas Rothe [7] did not find any enzyme activity using glycollate, glyoxylate or β-hydroxybutyrate as substrates, Davies and Davies [6] were able to estimate K_m values of these substrates for LDH isolated from potato tubers.

Inhibition by sulfhydryl reagents. Most dehydrogenases are –SH enzymes [12], and sulfhydryl groups have two roles in the function of LDH: they are essential to enzyme activity and are generally part of the active site; in addition they maintain the quaternary structure of

Table 1. Isolation of lactate dehydrogenase from pea seedlings

Fraction	Total activity (U)	Total protein (mg)	Specific activity (10^3 U/mg)	Degree of purification
Crude extract	7·42	3970	1·8	1·0
Protamine sulphate fractionation	7·16	2050	3·5	2·0
Ammonium sulphate fractionation	6·62	698	9·5	5
DEAE-cellulose chromatography	1·45	12	139	77
Sephadex G-150 chromatography	1·01	4	337	187

the molecule [12,13]. We have tested the effect of PCMB, NEMI and IA on the activity of LDH of pea plants. All these compounds acted as strong inhibitors of the enzyme. Thus LDH from pea plants behaves in a manner totally different from the enzyme of soybean, in which even prolonged incubation with PCMB, iodoacetate or arsenite in no way influences the activity of the enzyme [5]. LDH from pea plants was inhibited non-competitively by PCMB. The K_i value for NADH as substrate is $5{\cdot}85 \times 10^{-4}$ M and for pyruvate $9{\cdot}45 \times 10^{-4}$ M. Inhibition of the enzyme by NEMI and IA was irreversible; 50% inhibition was found with 5×10^{-3} and 10^{-2} M respectively. Furthermore, the protective influence of preincubating the enzyme with the coenzyme (NADH) and substrate (pyruvate) on the inhibition was tested. The results are summarized in Table 2. Preincubation of the enzyme with NADH as well as with pyruvate caused a decrease of inhibition in all cases. However, only protection of LDH activity by NADH against inhibition by PCMB was marked. Similar results have been obtained with animal LDH [14,15]. It may be assumed on the basis of these results that plant LDH also contains a sulfhydryl group in its active site, and that this group takes part in the formation of the binary enzyme–coenzyme complex, which is the first step in the LDH-catalysed reaction.

The markedly weaker effect of the coenzyme as well as the substrate on the inhibition of LDH by NEMI or IA may be explained as follows: these inhibitors are preferentially bound to the structural –SH groups which are not essential to enzyme activity. Preincubation of the enzyme, particularly with the coenzyme, stabilizes the quaternary LDH structure [16]. Evidently conformational changes are involved, which strengthen the bond between the subunits and hinder dissociation of the enzyme. It may be assumed that in the structure of the enzyme–coenzyme complex, –SH groups are less accessible to alkylating agents.

MW of LDH from pea seedlings. Gel filtration on a column of Sephadex G-150 and the comparison of elution volumes of our enzyme with those of proteins of known MW showed that the MW of LDH from pea plants is *ca* 145500. This value is very close to values obtained by other authors for the animal enzyme [12,17] and for the enzyme from soybeans [10].

EXPERIMENTAL

Pea plants, germinating in H_2O in Petri dishes at 25° for 48 hr served as a source of the enzyme.

Enzyme purification. Plant material was cooled and homogenized with 1 vol of 10 mM Tris-acetate buffer pH 7·4 containing 10^{-3} M EDTA, 10^{-3} M 2-mercaptoethanol and 0·1% Triton X-100. The homogenate was centrifuged for 30 min at 12000*g* to give supernatant (crude extract). A 2% protamine sulphate soln was added to the crude extract dropwise up to final concn of 0·16%. Precipitated proteins were removed by centrifugation, and the supernatant was then purified by fractionation with $(NH_4)_2SO_4$. The main portion of LDH activity precipitated between saturation levels of 30 and 40%. This fraction was desalted on a column of Sephadex G-25 and then chromatographed on DEAE-cellulose. A maximum of 200 mg of protein was applied to an ion-exchange column (35 × 1·5 cm), equilibrated with 10 mM Tris–OAc buffer pH 6·5. Proteins bound to the column were eluted with 300 ml Tris–OAc buffer pH 6·5 with Tris concentration rising linearly from 0·01 to 0·5 M, at a rate 3 ml per 10 min. The fractions containing active LDH, which was eluted in the 0·2–0·3 M Tris range, were combined, dialysed, concentrated by means of lyophilization and then subjected to gel filtration on Sephadex G-150 (column 90 × 3 cm). The column was equilibrated with 10 mM Tris–OAc buffer pH 6·5 which was also used to elute LDH. The eluate fractions containing the active enzyme were combined, dialysed and lyophilized. This enzyme preparation was used for all the following measurements. Protein concentration was determined by method in ref. [18].

Determination of enzyme activity. The rate of LDH-catalysed reaction in the direction of lactate oxidation was determined by means of the "Sevac-test-LDH" which is based on colorimetric measurement of the rate of pyruvate formation. For the reaction rate determination in the opposite direction, i.e.

Table 2. Influence of preincubation of lactate dehydrogenase of germinating pea plants with NADH (A) and pyruvate (B) on the inhibition of enzyme by agents reacting with sulfhydryl groups

Inhibitor	Conc. (M)	% Inhibition: No preincubation	% Inhibition: 5 Min preincubation
(A)			
PCMB	10^{-4}	15·3	2·5
	10^{-3}	65·0	12·5
NEMI	10^{-3}	18·5	7·5
	10^{-2}	44·2	27·5
IA	10^{-3}	20·3	12·5
	10^{-2}	64·0	35·0
(B)			
PCMB	10^{-4}	6·9	3·3
	10^{-3}	46·3	35·5
NEMI	10^{-3}	16·2	6·5
	10^{-2}	65·6	38·7
IA	10^{-3}	13·8	9·7
	10^{-2}	58·7	45·2

pyruvate reduction, the decrease of NADH concentration was measured at 366 nm in a reaction mixture containing 50 mM Tris–OAc buffer pH 7·2, 10^{-3} M Na pyruvate, and $1·2 \times 10^{-4}$ M NADH.

Inhibition of LDH. To study the function of –SH groups soln of 10 mM PCMB, 0·1 M NEMI and 0·1 M IA were used as inhibitors. K_i values for PCMB were determined for two inhibitor concentrations (10^{-4} and 10^{-3} M) from the reaction rate *vs* substrate concn relationship (either pyruvate or NADH).

Preincubation with substrate. The reaction medium, containing 500 μl Tris–OAc buffer pH 7·2 (0·1 M), 75 μl 10 mM Na pyruvate, 50 μl enzyme and 125 μl H_2O was incubated for 5 min. After preincubation, 10 or 100 μl of inhibitor soln was added and the reaction was started by addition of 200 μl of 0·4 mM NADH. Samples which were not preincubated with the substrate and samples containing no inhibitor were tested as controls.

MW was determined by gel filtration on a column of Sephadex G-150 as described by Andrews [19] using lysozyme (MW 14 500), bovine serum albumin (67 000), aldolase (147 000) and porcine γ-globulin (167 000) as standards.

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