ISOLATION AND PROPERTIES OF PLANT LACTATE DEHYDROGENASE

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Lactate dehydrogenase (E.C. 1.1.1.27) was isolated from soy beans (*Glycine* L.) by fractionation with ammonium sulfate, chromatography on DEAE-cellulose, and gel filtration. An electrophoretically homogeneous enzyme preparation was obtained, whose specific activity was 217-times higher than the activity of the extract. Some properties of the isolated enzyme were investigated. The dependence of the rate of the enzymatic reaction on substrate concentration shows a sigmoid profile. Certain intermediates of the citric acid cycle act as allosteric effectors. The pH-optimum of the reaction is 9·0 for pyruvate formation and 7·2 for lactate formation. The molecular weight of the isolated enzyme is approximately 140 000.

In certain plants, lactate is accumulated[1–4] during the germination, *i.e.* under relatively anaerobic conditions, and simultaneously changes also the specific activity of lactate dehydrogenase[5,6] which attains a maximum during the first days of the germination and then decreases. Lactate dehydrogenase catalyzes the following reversible reaction:

$$\text{pyruvate} + \text{NADH} \rightleftarrows \text{lactate} + \text{NAD}\,.$$

It can thus catalyze both the synthesis and the degradation of lactate and its role is very important during the first phase of plant germination when lactate is first formed and then again degraded.

The same enzyme of animal origin has been studied in more detail and it has been shown that its function is closely related to its structure. The character of lactate dehydrogenase is that of a heteromerous tetramer: it consists of two types of subunits four of which linked together give the active form[7,8]. The subunits have been designated H and M with respect to their occurrence in the organism. This structure gives the possibility of existence of five isozymes, H_4, H_3M through M_4. Different tissues of one individual show a characteristic distribution of lactate dehydrogenase isozymes. Isozymes with H subunits, which catalyze the conversion of pyruvate into lactate and are inhibited by higher concentrations of pyruvate, are predominant in aerobic tissues, such as, *e.g.* the heart. Isozymes with M subunits are localized predominantly in skeletal muscles and are not significantly inhibited by excess of pyruvate[9–11]. The isozyme composition of lactate dehydrogenase in the given tissue can vary, however, also with the external conditions, *e.g.* under anaerobiosis[12–14] and also during the development of the fetus[8,15] or during malignant growth[12].

By contrast, the knowledge of plant lactate dehydrogenase in this respect is extremely meagre. The main obstacle in the way to the elucidation of these problems is pre-

dominantly the lack of the plant enzyme in sufficiently pure form and adequate quantities. In this study, the isolation of lactate dehydrogenase from soy beans is presented. An electrophoretically homogeneous preparation of the enzyme was obtained. Some of its basic characteristics and the influence of effectors on the character of the conversion of pyruvate into lactate were studied.

EXPERIMENTAL

Extraction of enzyme. Unless stated otherwise, Tris-acetate buffer containing 10^{-3}M-EDTA and 10^{-4}M 2-mercaptoethanol were used in all operations with the plant enzyme. The enzyme was extracted from soy bean seeds which had been allowed to swell 32 h in water. The seeds were homogenized in a blender, 5 min in the cold, with 0·01M buffer at pH 7·4 to give a 50% homogenate. The latter was pressed through four layers of gauze and centrifuged 30 min at 12000 *g* in the cold. The supernatant was again passed over two layers of gauze to remove the lipid layer which had formed on the surface.

Purification of enzyme. The enzyme extract (30 ml) was desalted on a column of Sephadex G-25 (4 . 70 cm), equilibrated in 0·01M buffer at pH 7·4. The desalted solution was fractionated by precipitation with ammonium sulfate. The fraction precipitated at 20—33% saturation was treated further. This fraction was desalted first on a Sephadex G-25 column (2 × 40 cm) in batches of 200—250 mg of protein. The active fractions were then directly subjected to chromatographic separation on a DEAE-cellulose DE-50 column (1·5 × 45 cm). The maximum quantity of protein applied to this column was 200 mg. The column was equilibrated in 0·01 M buffer at pH 6·5; the same buffer at pH 6·5 and a linearly increasing concentration of Tris (0·01—0·5M) were used for the elution. The effluent was collected as 150 fractions 3 ml in volume. Lactate dehydrogenase was eluted by 0·15—0·18M buffer, *i.e.* in fractions 75—92.

The fractions showing the highest activity (78 through 89) were pooled, dialyzed against 0·001M buffer at pH 6·5 overnight, and freeze-dried. The protein (30 mg) was applied to a column of Sephadex G-150 (1 × 100 cm) equilibrated in 0·01M buffer at pH 6·5. The proteins were eluted from the column also by this buffer and collected as 3 ml fractions. The active fractions were pooled, and freeze-dried. This preparation of plant lactate dehydrogenase was used for the subsequent measurements.

Determination of enzymatic activity. The activity of lactate dehydrogenase as regards its catalysis of lactate oxidation was determined by the colorimetric test developed for the determination of the enzyme activity in the serum supplied by Imuna[16]. This test is based on the determination of the pyruvate which gives a colored hydrazone with 2,4-dinitrophenylhydrazine in alkaline media. One activity unit (U) is the amount of the enzyme which catalyzes the oxidation of 1 μmol of lactate per 1 min under these conditions.

The activity of the enzyme in certain measurements was determined also in terms of pyruvate reduction: the rate of decrease of NADH concentration at 366 nm was measured in Spekol spectrophotometer equipped with an amplifier and recorder. The composition of the reaction mixture was the following: 0·1M Tris acetate buffer pH 7·2, 5 . 10^{-4}M sodium pyruvate, and 4 . 10^{-5}M-NADH. An activity unit is defined as the amount of enzyme causing the optical density change by 1 in 1 min.

Protein concentration was determined by the method of Lowry and coworkers[17].

Polyacrylamide gel electrophoresis was carried out by the method of Feuer and Lynch[18] using the modification of Barth and coworkers[19].

Molecular weight determinations were carried out according to Andrews[20] using the standards given in Fig. 1.

TABLE I

Purification of Lactate Dehydrogenase from Soy Beans

The values in the Table are based on 100 g of starting material.

Fraction	Total activity U	Proteins mg	Specific activity $U/mg \cdot 10^{-3}$	Degree of purification
Extract	6·57	1 025	6·4	1
20—33% saturation with $(NH_4)_2SO_4$	2·10	112	18·4	3
Effluent from DEAE-cellulose column	1·86	3·6	516·0	80
Effluent from Sephadex G-150 column	1·63	1·2	1 390·0	217

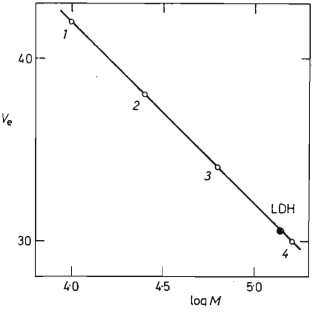

FIG. 1

Determination of Molecular Weight of Lactate Dehydrogenase from Soy Beans by Gel Filtration

Dependence of elution volume V_e (ml) on the logarithm of molecular weight: *1* cytochrome c, *2* trypsin, *3* bovine serum albumin, *4* pig γ-globulin.

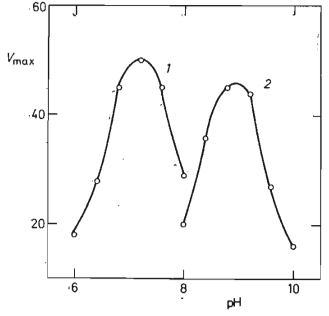

FIG. 2

Dependence of Maximum Reaction Rate V_{max} $(U \cdot 10^{-2})$ for Lactate Dehydrogenase from Soy Beans on pH as Regards Reduction of Pyruvate *1* and Oxidation of Lactate *2*

RESULTS

Purification of Alcohol Dehydrogenase from Soy Beans

We used the combination of several methods, *i.e.* fractionation by ammonium sulfate, chromatography on DEAE-cellulose, and gel filtration and were able to obtain a preparation of plant lactate dehydrogenase whose specific activity was 217-times higher than that of the original extract (Table I). The final enzyme preparation was perfectly homogeneous when subjected to chromatography on Sephadex G-150 and polyacrylamide–gel electrophoresis. The frozen or freeze-dried preparation of lactate dehydrogenase prepared by this procedure retains unaltered activity for 7 days.

Properties of Lactate Dehydrogenase from Soy Beans

We determined the molecular weight of the enzyme by gel filtration on Sephadex G-150 by comparison with standards of known molecular weight. The molecular weight of lactate dehydrogenase from soy beans was found to be 140 000 (Fig. 1).

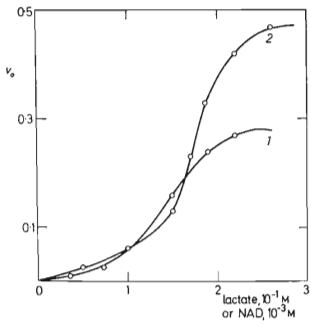

FIG. 3

Dependence of Initial Rate of Reaction Catalyzed by Lactate Dehydrogenase from Soy Beans, v_0(U . 10^{-1}) on Concentration *1* of Lactate at Constant Concentration of NAD ($1{\cdot}5 . 10^{-3}$M) and *2* of NAD at Constant Concentration of Lactate ($1{\cdot}5 . 10^{-1}$M)

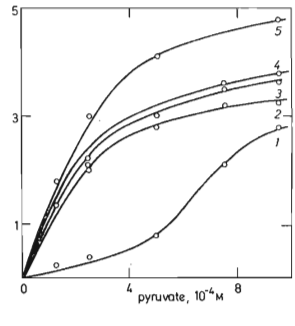

FIG. 4

Dependence of Initial Rate of Reaction Catalyzed by Lactate Dehydrogenase from Soy Beans, v_0(U . 10^{-2}) on Concentration of *1* Pyruvate in the Presence of Effectors, *2* Malate (5 . 10^{-5}M), *3* Succinate (5 . 10^{-5}M) *4* α-Ketoglutarate (5 . 10^{-5}M), and *5* Citrate (5 . 10^{-5}M)

TABLE II

Influence of Effectors on Activity of Lactate Dehydrogenase from Soy Beans
The degree of the activation is expressed by a comparison of V_{max}-values.

Effector	Activation of enzyme, %	
	pyruvate	NADH
Malate	9·5	14·0
Succinate	22·5	—
α-Ketoglutarate	30·7	3·8
Citrate	61·2	3·8

The pH-optimum of the reaction catalyzed by lactate dehydrogenase was determined from the dependence of V_{max} on pH. The pH-optimum of activity of the enzyme prepared from soy beans lies at pH 9·0 as regards the oxidation of lactate and at pH 7·2 as regards the reduction of pyruvate to lactate (Fig. 2).

The profile of the plot of the reaction rate versus substrate concentration is not hyperbolic either for the lactate or for the pyruvate; its character is that of a sigmoid curve and the dependence does not therefore follow the Michaelis–Menten kinetics. We present therefore the apparent K_m-values for the individual substrates determined as substrate concentrations corresponding to half the maximum reaction rate. These values are $1{\cdot}4 \cdot 10^{-1}$M for lactate, $1{\cdot}7 \cdot 10^{-3}$M for NAD, $6{\cdot}8 \cdot 10^{-4}$M for pyruvate, and $3{\cdot}4 \cdot 10^{-5}$M for NADH.

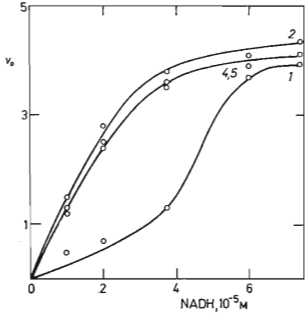

FIG. 5

Dependence of Initial Rate of Reaction Catalyzed by Lactate Dehydrogenase from Soy Beans, v_0(U . 70^{-2}) on concentration of *1* NADH in the Presence of Effectors, *2* Malate ($5 \cdot 10^{-5}$M), *4* α-Ketoglutarate ($5 \cdot 10^{-5}$M), and *5* Citrate ($5 \cdot 10^{-5}$M)

We also found that the citrate, α-ketoglutarate, succinate, and malate act as allosteric effectors of this enzyme: the character of the saturation curve of the enzyme changed from sigmoid to hyperbolic in their presence. All these compounds activated the lactate dehydrogenase from soy beans. The maximum increase of the activity of the enzyme as regards pyruvate was brought about by citrate (by 60%), whereas the maximum increase of its activity as regards NADH was caused by the presence of malate (by 14%, Fig. 3–5), (Table II).

DISCUSSION

Lactate dehydrogenase obviously plays two roles in the germinating plants: it catalyzes the formation of lactate from pyruvate during the early phase of germination in the period of relative anaerobiosis and it catalyzed the degradation of lactate after the testa has ruptured and aerobic conditions in the plant have been established. In animal tissues, these two reactions are probably catalyzed by two different types of lactate dehydrogenase[9–11]. The enzymes specializes for aerobic or anaerobic metabolism differ also in the structure of their molecules and in their properties. By contrast, very little is known about the function, structure and properties of the plant enzyme.

We made an effort to purify and to characterize lactate dehydrogenase from soy beans and thus to contribute to the knowledge of the role of this enzyme in the metabolism of germinating plants. Soy beans were used as starting material at that phase of germination when the specific activity of the enzyme is maximal, *i.e.* after 32 hours of germination. We were able to prepare by the above described procedure the enzyme which was perfectly homogeneous when subjected to gel filtration and polyacrylamide gel electrophoresis. Its specific activity was approximately 220-times higher than the activity of the original extract. The so far most successful effort to isolate lactate dehydrogenase from plant material represent the experiments of King[6] who obtained a 110-times enriched inhomogeneous preparation with many times lower specific activity from soy beans.

We used the pure, electrophoretically homogeneous enzyme to study its interaction with substrates or cofactors and intermediates of the citric acid cycle. The dependence of the conversion rate of the substrates of lactate dehydrogenase, *i.e.* of lactate pyruvate, NAD, and NADH on their concentration shows a sigmoid-shaped profile. This profile becomes hyperbolic for pyruvate and NADH in the presence of certain intermediates of the citric acid cycle. This indicates an allosteric transition[21]. Lactate dehydrogenase is activated by citrate, α-ketoglutarate, malate, or succinate and the apparent K_m-value both for pyruvate and for NADH is decreased at the same time. The occupation of the allosteric site by these intermediates obviously leads to an increase of the affinity of the enzyme for its own substrate.

It would appear from a comparison of the properties of the plant and the animal enzyme that lactate dehydrogenase from soy beans behaves in the anaerobic phase very similar to the animal M_4 isozyme, localized predominantly in skeletal muscles. This enzyme is known[11] to catalyze rapid conversion of pyruvate into lactate; it is not inhibited by excess of pyruvate. It acts therefore predominantly as pyruvate reductase at the site of intensive anaerobic metabolism[10]. It could be deduced from this analogy that in germinating seeds predominates in the period of anaerobic metabolism lactate dehydrogenase in the form of pyruvate reductase and that it plays a regulatory role in those plant tissues in which intensive anaerobic glycolysis takes place.

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