SOME STRUCTURAL AND KINETIC CHARACTERISTICS OF LACTATE DEHYDROGENASE FROM SOY-BEAN SEEDLINGS (*Glycine max*. L.)

J. BARTHOVÁ, P. HRBAS and S. LEBLOVÁ

Department of Biochemistry,
Charles University, 128 40 Prague 2

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The paper reports on some kinetic and structural characteristics of lactate dehydrogenase (E.C. 1.1.1.27), isolated from soy-bean seedlings by a procedure involving preparative polyacrylamide gel electrophoresis as a final step. The reaction catalyzed by soy-bean lactate dehydrogenase is inhibited competitively in both directions by certain coenzyme analogs; the enzyme catalyzes also the conversion of other α-hydroxy and α-keto acids. The subunit molecular weight and the number of sulfhydryl groups in the enzyme molecule were also determined.

The present knowledge of animal lactate dehydrogenase is extensive[1-3] indeed and it contrasts strikingly with the meager information on the same enzyme from plants. James and Cragg[4] found the active enzyme in germinating barley, Sherwin and Simon[5] in bean seeds, Davies and Davies[6] extracted the enzyme from potato tubers and demonstrated that it is NAD-specific. King[7] isolated active lactate dehydrogenase from soy-beans and showed that the enzyme was L-lactate- and NAD-specific. Soy-bean lactate dehydrogenase catalyzed also reduction of hydroxypyruvate and glyoxalate. This author reported that compounds reacting with sulfhydryl groups were without any effect on the activity of the enzyme. In our preceding paper[9], we used a partly purified enzyme from soy-beans to study some of its properties, such as, *e.g.* the dependence of reaction rate on substrate concentration, the influence of effectors on enzyme activity, the pH-optimum of the enzyme, its molecular weight *etc.*

In this study, we were able to prepare highly active and electrophoretically homogeneous lactate dehydrogenase by polyacrylamide gel electrophoresis. The quality of this enzyme preparation permitted us to study additional kinetic and structural characteristic of the enzyme from plants.

EXPERIMENTAL

Material

Source of enzyme: soy-beans (*Glycine max* L.) which were allowed to swell 32 h in distilled water.

Chemicals used: Tris(hydroxymethyl)aminomethane (Tris), A.R., NADH (Merck, AG, Darmstadt), 2-mercaptoethanol, NAD (Koch-Light, England), sodium pyruvate, A.R., 2,4-dinitrophenylhydrazine, pure (Lachema, Brno), Sevac LDH test (Immuna, Šarišske Michalany), LDH-L Stat-Pack (Calbiochem, USA), DEAE-cellulose (Whatman, England), Sephadex G-25 (Pharmacia, Uppsala). The remaining chemicals were of A.R. purity grade.

Methods

Determination of protein concentration was performed by the method of Lowry and coworkers[8].

The determination of lactate dehydrogenase activity was carried out (*a*) by the Sevac-LDH-test, *i.e.* by colorimetric measurement of the rate of pyruvate formation, (*b*) by the Calbiochem test, *i.e.* by spectrophotometric measurement of rate of NADH formation, and (*c*) in certain cases the enzyme activity was calculated from the rate of disappearance of NADH from a reaction medium containing 0·1M phosphate buffer at pH 7·2, 10^{-3}M sodium pyruvate, and $4 \cdot 10^{-5}$M-NADH. The quantity of enzyme catalyzing the conversion of 1 μmol of substrate per 1 minute was taken for one activity unit.

Isolation of enzyme. The initial stages of the process were identical to those of the method used earlier[9]. The active portion, eluted from the DEAE-cellulose column, was dialyzed against 0·001M Tris-acetate buffer at pH 7·4 and fractionated by preparative electrophoresis according to Jovin and coworkers[10] in Poly-Prep-100 Apparatus (Buchler Instrument division). Samples of 80 mg of proteins in 18% sucrose solutions were applied onto a column approximately 10 cm high. The proteins were concentrated first at the top of the column at 30 mA for half-an-hour. The fractionation itself was allowed to proceed 15 h at 70 mA. The inner and outer gel surface was maintained at 0—2°C during the separation. Fractions of 3 ml were collected every 15 min.

Disc electrophoresis was carried out by the method of Feuer and Lynch[11]; the modification described by Barth and coworkers[12] was used. Disc electrophoresis in the presence of sodium dodecyl sulfate[13] was used to check the subunit structure of the enzyme. Bovine serum albumin (molecular weight 62000), ovalbumin (46500), aldolase (40000), trypsin inhibitor (21550), and ribonuclease (18500) were used as standards.

Sulfhydryl groups were determined according to Sedlak and Lindsay[14]. Cysteine served as a standard.

RESULTS

Isolation of lactate dehydrogenase. We used fractionation with ammonium sulfate, chromatography on DEAE-cellulose, and preparative electrophoresis in polyacrylamide gel and were able to obtain a preparation of lactate dehydrogenase, whose specific activity was 1160-times higher than the activity of the crude extract. The enzyme preparation obtained was homogeneous electrophoretically and also when subjected to gel filtration on Sephadex G-150 (Table I, Fig. 1).

Determination of Michaelis constants. We found that the kinetic behavior of the enzyme is, among others, also a function of enzyme concentration[15]. The hyperbolic profile of the plot of reaction rate *versus* substrate concentration becomes sigmoid at lactate dehydrogenase concentrations higher than 25 mU/ml. Nevertheless, the

K_m-values determined according to Lineweaver and Burk and the values of apparent $K_m(S_{0.5})$, determined for higher enzyme concentrations as substrate concentrations corresponding to half the maximum reaction rate, do not differ any significantly (Table II).

Inhibition of lactate dehydrogenase by coenzyme analogs. The inhibitory effect of compounds in the series adenine, adenosine, AMP, ADP, ATP affects both directions of the reaction catalyzed by soy-bean lactate dehydrogenase and competes with NAD and NADH, respectively. The inhibitory constants K_i are given in Table III.

Substrate specificity of lactate dehydrogenase. Lactate dehydrogenase catalyzed the conversion of also other α-hydroxy and α-keto acids. The K_m-values characterizing individual substrates are compared in Table IV.

Subunit structure of soy-bean lactate dehydrogenase. The molecule of the enzyme, whose molecular weight is 140000 according to our earlier data[9], dissociates to subunits of identical molecular weight and electrophoretic mobility in sodium dodecyl sulfate solution. A comparison of the relative mobilities of the standards with the mobility of our enzyme preparation shows that the subunit molecular weight is 36000.

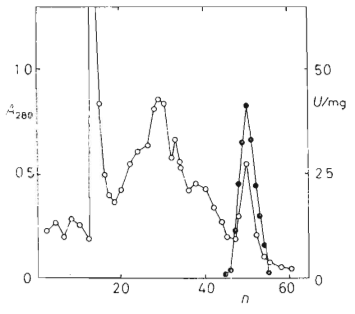

FIG. 1

Separation of Prepurified Preparation of Lactate Dehydrogenase form Soy Bean Seedlings by Preparative Electrophoresis in Polyacrylamide Gel

○—○ A_{280}, ●—● specific activity of lactate dehydrogenase (U/mg)

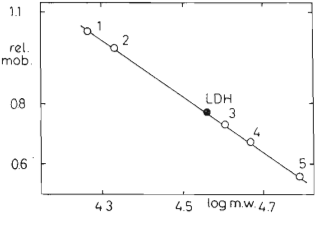

FIG. 2

Determination of Molecular Weight of Subunits form Soy-Bean Lactate Dehydrogenase by Electrophoresis in Sodium Dodecyl Sulfate Solution

Dependence of relative mobility of compounds on logarithm of molecular weight. *1* Ribonuclease, *2* trypsin inhibitor, *3* aldolase, *4* ovalbumin, *5* bovine serum albumin.

Content of sulfhydryl groups in molecule of lactate dehydrogenase. We found that the concentration of sulfhydryl groups in our sample was $1{\cdot}7 \cdot 10^{-5}$M. On condition that the molecular weight of soy-bean lactate dehydrogenase is 140000, one molecule of the enzyme contains 24 sulfhydryl groups.

DISCUSSION

We used the procedure described earlier[9], complemented by preparative electrophoresis in polyacrylamide gel, and were able to obtain a preparation of lactate dehydrogenase from soy-bean seedlings. The specific activity of this preparation was

TABLE I

Isolation of Lactate Dehydrogenase from Soy-Bean Seedlings
The values in the table are based on 500 g of starting material.

Fraction	Total activity U	Proteins mg	Specific activity ml/mg	Degree of purification
Extract	46·75	20 400	2·29	1
30—40% Saturation with ammonium sulfate	30·40	4 550	6·65	2·9
Effluent from DEAE-cellulose	12·60	84	150·0	65
Preparative electrophoresis	5·56	2·1	2 643·0	1 160

TABLE II

Values of Michaelis Constants (K_m) and of Apparent Michaelis Constants ($S_{0.5}$) of Soy-Bean Lactate Dehydrogenase

Substrate	K_m, mol/l	$S_{0.5}$, mol/l
Pyruvate	$6{\cdot}3 \cdot 10^{-4}$	$6{\cdot}8 \cdot 10^{-4}$
NADH	$1{\cdot}4 \cdot 10^{-5}$	$3{\cdot}4 \cdot 10^{-5}$
Lactate	$3{\cdot}5 \cdot 10^{-2}$	$1{\cdot}4 \cdot 10^{-1}$
NAD	$3{\cdot}1 \cdot 10^{-4}$	$1{\cdot}7 \cdot 10^{-3}$

1160-times higher than the enzyme activity of the crude extract. The effect of the electrophoretic separation was a 18-fold increase of the specific activity of the enzyme preparation.

The K_m-values, characterizing individual substrates of the enzyme from soy-beans (Table II) can be compared with the corresponding data on lactate dehydrogenase of animal origin[16-20] or that of potatoes[6]. These values essentially do not differ. A fact deserving interest is that the kinetic behavior of the enzyme from soy-bean seedlings depends not only on the substrate concentration but is a function of the enzyme concentration, too. The hyperbolic character of the dependence of substrate turnover rate on substrate concentration becomes sigmoid if the enzyme concentration is higher than 25 mU/ml. The allosteric character of lactate dehydrogenase of animal origin has been demonstrated by several authors[20-22]. Markert and Massa-

TABLE III

Inhibition of Soy-Bean Lactate Dehydrogenase

Inhibition constants for compounds analogous to the coenzyme.

Inhibitor	K_i, mol/l reaction	
	reduction of pyruvate	oxidation of lactate
Adenine	$7{\cdot}5 \cdot 10^{-4}$	$3{\cdot}4 \cdot 10^{-3}$
Adenosine	$2{\cdot}6 \cdot 10^{-4}$	$2{\cdot}3 \cdot 10^{-3}$
AMP	$9{\cdot}6 \cdot 10^{-4}$	$4{\cdot}4 \cdot 10^{-3}$
ADP	$2{\cdot}7 \cdot 10^{-6}$	$1{\cdot}2 \cdot 10^{-5}$
ATP	$5{\cdot}6 \cdot 10^{-7}$	$2{\cdot}5 \cdot 10^{-6}$

TABLE IV

Specific Activity of Soy-Bean Lactate Dehydrogenase

K_m-Values for certain α-hydroxy and α-keto acids.

Substrate	K_m, mol/l	Substrate	K_m, mol/l
Lactate	$3{\cdot}5 \cdot 10^{-2}$	Pyruvate	$6{\cdot}3 \cdot 10^{-4}$
Malate	$1{\cdot}2 \cdot 10^{-1}$	α-Ketoglutarate	$8{\cdot}7 \cdot 10^{-3}$
Glycolate	$3{\cdot}8 \cdot 10^{-2}$		

ro[23] explain the allosteric behavior of the enzyme by assuming that the enzyme dissociates to the dimer at lower concentration and that an association–dissociation equilibrium between the dimer and the tetramer is attained; the latter could serve as a molecular basis of the allosteric behavior of the enzyme. Monod[24] and Koshland[25;26] on the contrary assume that conformational changes in the structure of the subunits[27] are responsible for the allosteric character of the enzyme. It appears that lactate dehydrogenase from soy-bean seedlings shows a sigmoid saturation curve in case that its allosteric site is not occupied (*i.e.* at a higher enzyme concentration and thus at a relative substrate deficiency). After the allosteric site has been occupied either by the substrate (at a lower enzyme concentration and thus at a relative substrate excess) or by some effectors, *e.g.* by the citrate cycle[9] intermediates, the curve assumes a hyperbolic profile.

Compounds analogous to the coenzyme inhibited the activity of soy-bean lactate dehydrogenase. The inhibition is competitive both with respect to NAD and NADH; the K_i-value decreases in the series adenine, AMP, ADP, and ATP. Appearingly the presence of phosphate or alternatively of pyrophosphate in position 5′ plays an important role in the inhibition mechanism and hence also in the mechanism of binding of both the inhibitors and also of the coenzyme itself to the enzyme. This is indicated also by the finding that the K_i-values for adenine and adenosine are at least by one order higher than the corresponding values for their phosphates.

Lactate dehydrogenase from soy-bean seedlings catalyzes also the conversion of α-hydroxy and α-keto acids other than lactate and pyruvate. An interesting fact which follows from the K_m-values is that the affinity for glycolate is almost as high as the activity for lactate. A typical feature of germinating seeds, especially of lipid seeds, is a high activity of the glyoxalate cycle; it is quite probable that even lactate dehydrogenase can participate on the conversion of lipids into carbohydrates at this stage of growth.

We have shown earlier[9] by the method of gel filtration that the molecular weight of lactate dehydrogenase from soy-beans is 140000. The enzyme molecule dissociates in sodium dodecyl sulfate to subunits of equal molecular weight, *i.e.* 36000. It is therefore probable that lactate dehydrogenase from soy beans has the structure of a tetramer, similarly to most lactate dehydrogenases of animal origin. Similarly the number of sulfhydryl groups, *i.e.* 24, in the molecule of soy-bean lactate dehydrogenase is approximately the same as in the enzymes of animal origin[2,28]. It is known that SH--groups can play two roles in the molecule of lactate dehydrogenase. First, they are involved in maintaining the three-dimensional structure of the protein molecule and second, each subunit contains at least one SH-group essential for the formation of a ternary enzyme–coenzyme-substrate complex. An investigation of the functions of individual sulfhydryl groups in the molecule of soy-bean lactate dehydrogenase is in progress.

REFERENCES

1. Cahn R. D., Kaplan N. O., Levine L., Zwilling E.: Science *136*, 962 (1962).
2. Holbrook J. J., Stinson R. A.: Biochem. J. *120*, 289 (1970).
3. d'A. Heck H.: J. Biol. Chem. *244*, 4375 (1969).
4. James W. O., Cragg J. M.: New Phytologist *42*, 28 (1943).
5. Sherwin T., Simon E. W.: J. Exptl. Botany *20*, 776 (1969).
6. Davies D. D., Davies S.: Biochem. J. *129*, 831 (1972).
7. King J.: Can. J. Botany *48*, 533 (1970).
8. Lowry H. O., Rosebrough N. J., Farr H. L., Randal R. J.: J. Biol. Chem. *193*, 265 (1951).
9. Barthová J., Hrbas P., Leblová S.: This Journal *38*, 2174 (1973).
10. Jovin T., Chrambach A., Naughton M. A.: Anal. Biochem. *9*, 351 (1964).
11. Feuer H., Lynch U. E.: J. Am. Chem. Soc. *75*, 5027 (1953).
12. Barth T., Nedkov P., Rychlík I.: This Journal *35*, 3133 (1970).
13. Maizel T. V. F.: Science *151*, 988 (1966).
14. Sedlak J., Lindsay R. H.: Anal. Biochem. *75*, 192 (1968).
15. Hrbas P.: *Thesis*. Charles University, Prague 1973.
16. Winer A. D., Schwert G. W.: J. Biol. Chem. *231*, 1065 (1958).
17. Pesce A., McKay R. H., Stolzenbach F., Cahn R. D., Kaplan N. O.: J. Biol. Chem. *239*, 1732 (1964).
18. Pesce A., Fondy T. P., Stolzenbach F., Castillo T., Kaplan N. O.: J. Biol. Chem. *242*, 2151 (1967).
19. Geyer H.: Hoppe-Seyler's Z. Physiol. Chem. *348*, 823 (1967).
20. Hathaway G., Cridle R. S.: Proc. Natl. Acad. Sci. US *56*, 680 (1966).
21. Fritz J. P.: Science *150*, 364 (1965).
22. Niesselbaum J. S., Bodansky O.: J. Biol. Chem. *236*, 323 (1961).
23. Markert C. L., Massaro E. J.: Science *162*, 695 (1968).
24. Monod J., Wyman J., Changeux J. - P.: J. Mol. Biol. *12*, 88 (1966).
25. Koshland, D. E. jr, Némethy G., Filmer D.: Biochemistry *5*, 365 (1966).
26. Koshland D. E. jr, Neet K. E.: Ann. Rev. Biochem. *37*, 359 (1968).
27. Fruhaufová L.: Československ. fysiol. *20*, 13 (1971).
28. DiSabato G., Kaplan N. O.: Biochemistry *2*, 776 (1963).

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