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AFFINITY CHROMATOGRAPHIC SEPARATION OF PLANT LACTATE DEHYDROGENASE

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato tuber; plant LDH isoenzymes; affinity chromatography; MW; SDS electrophoresis.

Abstract—Lactate dehydrogenase from potato tubers was purified by the use of several standard purification procedures as well as by affinity chromatography on Cibacron Blue F3G-A Sepharose and the separation behaviour of the enzyme and its multiple molecular forms was compared with the isoenzyme pattern of mammalian lactate dehydrogenases. The plant LDH isoenzymes were found to be charge isomers having a MW of 84500, 106000 and 115000 for isoenzyme 1, 2 and 3, respectively. On SDS-electrophoresis two different subunits were found having a MW of 41000 and 43000, respectively.

INTRODUCTION

Under anaerobic conditions, pyruvate is reduced to lactate by the enzyme lactate dehydrogenase (LDH), and while lactic acid accumulates, NAD which serves as a cosubstrate in this reaction is consumed within the glycolytic pathway. The production of lactate has been demonstrated not only in bacteria and animals, but also in several plants such as germinating seeds [1–4] and potato tubers [5, 6].

The properties and significance of LDH have been studied intensively in animals and adequately reviewed [7, 8] but information about the plant enzyme is still scanty. Within the plant kingdom LDH has been found in germinating barley [9], bean seeds [2], soy-beans [10] and potato tubers [11]. While several attempts have been made to purify the plant enzyme by the use of standard purification procedures such as ammonium sulfate saturation, gel chromatography, ion exchange chromatography and electrophoresis [10–14], an affinity chromatographic method to purify the plant enzyme has not yet been described.

One of the striking features of mammalian LDH is its occurrence in multiple molecular forms (e.g. [15–19]). The isoenzymes of animal LDH have been classified as primary isoenzymes [20] according to their ability to produce a total of 5 isoenzymes of the two homopolymeric forms by random association of its 4 subunits [21]. Although the existence of LDH isoenzymes has also been demonstrated in several plants, legume roots [22] and potato tubers [14, 23, 24], its isoenzymic nature has still to be investigated. The present work describes a procedure to purify potato tuber LDH and its isoenzymes.

RESULTS

Influence of different extraction media on enzyme activity and number of isoenzymes

The activity of potato tuber LDH in crude extracts increased when Tris–acetate buffers were used instead of Pi buffers. With alkaline Tris-buffers more activity could be extracted than with neutral ones. During a longer period of storage at −16°, the enzyme lost significantly more activity in Pi buffers than in Tris-buffers.

Acid precipitation

Fig. 1 gives the results of a typical experiment in which we tried to precipitate non-LDH protein at acidic pH. The maximum sp. act. is reached when the solution is acidified to pH 4.6–4.8. The purification factor after acid precipitation was *ca* 2.2 (Table 1).

DEAE ion exchange chromatography

Several attempts were made to separate LDH on DEAE cellulose. This proved to be successful with a

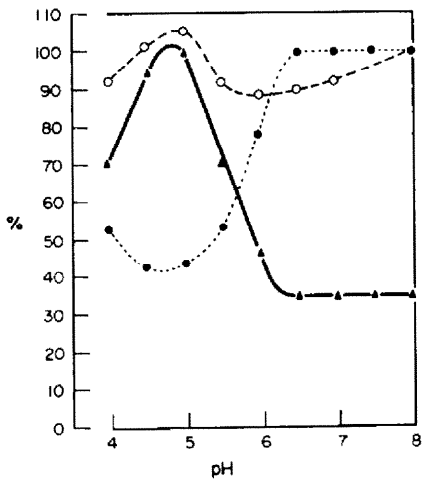

Fig. 1. Acid precipitation of non LDH proteins using a Sephadex G-25 extract of potato tubers. Plot of pH vs % of LDH activity in the supernatant after centrifugation. O—O % μkat LDH activity/l. ●–● % of protein content, ▲—▲ % of sp. act. (nkat LDH/g protein).

Table 1. Purification of lactate dehydrogenase from potato tubers

Procedure		Vol. (ml)	Activity (nkat/ml)	Total (nkat)	Protein (mg/ml)	Specific activity (μkat/g)	Yield %	Purification
Crude extract		266	10.3	2740	11.1	0.93	100	1.0
G 25 chromatography		372	5·9	2200	5.2	1.13	80.1	1.2
Acid precipitation		365	6.2	2260	3.0	2.06	82.6	2.2
DEAE chromatography		11 4	116	1330	9·6	12·1	48·4	13·0
Centriflo concentration		1 8	577	1040	52.8	10.9	37.9	11.7
Blue Sepharose chromatography		8.5	111	946	3.9	28.5	34.5	30.7
Centriflo and disc-electrophoresis	Iso 1	3.3	39.8	131	0·84	47.4	4.8	51.0
	Iso 2	3.0	19.2	57	0.64	30.0	2.1	32.2
	Iso 3	2.5	2.9	7	0.59	4.91	0.2	5.3

20–40% ammonium sulfate extract made from a crude extract of potato tubers. LDH eluted at *ca* 0.19 M KCl, while protein peaked at *ca* 0.09 M KCl. Separation of isoenzymes could not be demonstrated. If the slope of the gradient was decreased, the enzyme eluted at about 0.20 M KCl but also with one single peak.

A change in pH, saturation of ion exchanger with protein, or a change in the gradient volume did not alter the elution pattern of plant LDH significantly. However, if the combined fractions of the LDH peak were electrophoresed on polyacrylamide gels, 3 LDH isoenzymes could be separated. On DEAE ion exchange columns, LDH 1 from pig heart eluted at *ca* 0.19 M KCl which is the concentration for elution of potato LDH, while LDH 5 from pig or rabbit muscle eluted at *ca* 0.05 M KCl. Therefore, it seems that LDH 5 from animal sources has no similarity to the plant enzyme.

Affinity chromatography

In a further purification procedure, partially purified potato tuber LDH was chromatographed on Blue Sepharose C_L–6_B. The binding of potato tuber LDH to Blue Sepharose depends on pH. At pH 5, 40–50% more LDH was bound as compared to that at pH 7. If the enzyme was adsorbed at pH 7 it could be eluted neither by KCl up to 0.5 M nor by NAD or NADH up to a concentration of 3 mM. About 10% of LDH was desorbed at pH 5 when the affinity gel was treated with 0.5 M KCl. No enzyme eluted if the gel was treated with 3 mM NAD at the same pH value. LDH from potato tubers, which was adsorbed at pH 4.7 (0.01 M Tris–acetate buffer) to Blue Sepharose, could be released by a pH-gradient from pH 4.7 to 8.8 or by elution at pH 9. The recovery was *ca* 73% and the purification factor *ca* 2.6 (see Table 1). The capacity of Blue Sepharose for the plant enzyme was 60–70 μkat LDH activity/g of wet affinity gel.

Purification and electrophoresis

As can be seen from Table 1, LDH from potato tubers could be enriched to a sp. act. of 47 μkat/g protein (2840 mU/mg). After every purification step, 100 μl of enzyme solution was submitted to a polyacrylamide gel electrophoresis. Although no separation of isoenzymes could be observed in DEAE nor in affinity chromatography, up to 3 isoenzymes were separated when the protein solutions of each procedure were electrophoresed on polyacrylamide.

Electrophoretic comparison of plant and mammalian LDH isoenzymes

Comparing LDH isoenzymes 1 and 5 from pig and rabbit with potato LDH isoenzymes by histochemical assay the latter are very similar in their electrophoretic separation behaviour and appear on the electrophoretic horizon of mammalian LDH isoenzyme 1. No plant isoenzyme could be found having a similar electrophoretic mobility to animal LDH 5

Determination of MWs

The use of thin layer gel-chromatography on Sephadex G-100 according to the method of Jaworek [25] gave a MW for the plant LDH enzyme of *ca* 95 000. Purified isoenzyme 3 of potato tuber LDH was used for a SDS electrophoretic determination of the subunit MW of the enzyme. Two subunits were found having a MW of 41 000 and 43 000 respectively.

Determination of charge isomers

The separation behaviour of plant LDH isoenzymes 1, 2 and 3 was also investigated in polyacrylamide gels of 5 different acrylamide concentrations at a constant ratio of bismethylene acrylamide. A plot of the log of the electrophoretic movement (R_f) against per cent acrylamide concentration of potato LDH resulted in 3 convergent lines indicating that the 3 isoenzymes do not differ in their charge but in their MW (Fig. 2). This figure also gives the separation behaviour of glucose-6-phosphate dehydrogenase from yeast under the same conditions. Using this enzyme as a standard the following MWs were determined for the LDH isoenzymes: 1:84 500, 2:106 000 and 3:115 000.

DISCUSSION

The dye Cibacron blue F3G-A has been recently introduced as a so-called group-specific ligand [26]. The dye covalently bound to an agarose matrix served in binding studies of kinases and dehydrogenases [27] as well as in the final purification of phosphofructokinase [26], animal LDH and albumin [28]. Because the dye binds with a wide range of proteins [29], it is suggested that it is bound to those proteins which have as a super secondary structure, the so-called dinucleotide fold. It has been reported that the binding capacity of Cibacron blue

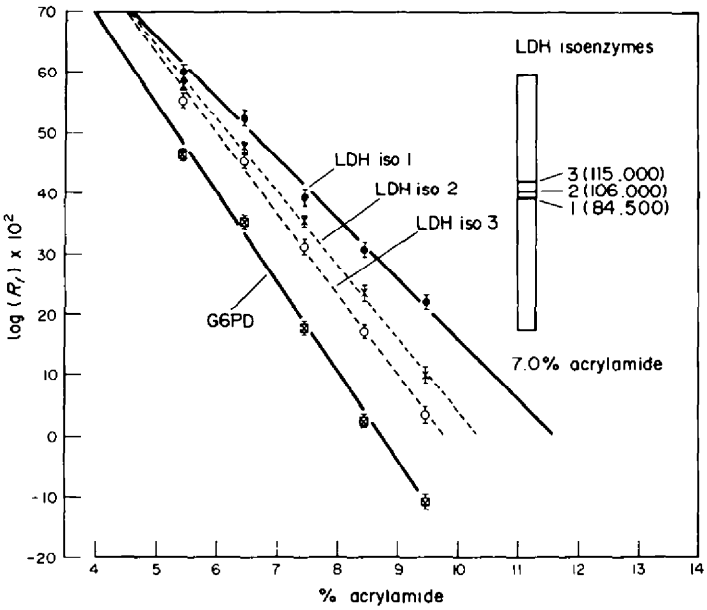

Fig. 2. Determination of the MWs of potato LDH isoenzymes 1, 2 and 3, respectively according to ref. [36] using glucose-6-phosphate as a standard. The lines for the LDH isoenzymes 1, 2 and 3 intersect at one common point indicating that these multiple molecular forms differ in their MWs but have the same charge.

F3G-A Sepharose for muscle and yeast phosphofructokinase is dependent on pH [30]. As could be shown in the present investigation, the binding capacity of Blue Sepharose for potato tuber LDH was also pH dependent. Although several animal enzymes from the glycolytic pathway were bound to the immobilized dye at neutral pH [27], the affinity gel adsorbed potato LDH best at *ca* pH 5. Animal LDH could be desorbed from Blue Sepharose at neutral pH and NaCl at a concentration of more than 100 mM [28]. On the other hand, we were not able to elute potato LDH either at neutral or at acid pH using NaCl at a concentration of up to 500 mM. Potato LDH could be desorbed from Blue Sepharose by the use of a pH-gradient ranging from 4.7 to 8.8. In this respect the enzyme behaves like glucose-6-phosphate dehydrogenase and LDH from animal sources bound to N^6-(aminohexyl)-5'-AMP-Sepharose which can be desorbed by a change in the pH of the elution solution [31].

As has been demonstrated in this work, potato tuber LDH eluted with only one peak when chromatographed on Blue Sepharose or on DEAE–cellulose. On the other hand, the enzyme could be separated into 3 active enzyme bands after each purification procedure when electrophoresed on polyacrylamide. Also starch gel electrophoresis resulted in the same isoenzyme pattern. This is in contrast to the mammalian LDH which can be separated into 5 isoenzymes when chromatographed on DEAE–cellulose [32–35] or when submitted to an electrophoretic separation [15, 16, 18].

In comparison with the animal LDH isoenzymes, the plant multiple molecular forms showed smaller differences in their electrophoretic mobility. Potato LDH isoenzyme 1, 2 and 3 and pig LDH isoenzyme 1 migrated almost to the same position. Because the plant LDH isoenzymes cannot be separated by DEAE–cellulose ion exchange or by affinity chromatography on Blue Sepharose, it may be expected that the differences in the net charges of the 3 electrophoretically separated isoenzymes are too small to be used for a separation with these methods. In accordance with this assumption we found that the plant isoenzymes belong to the group of the charge isomers having different MWs. This is in contrast to the mammalian isoenzymes which are size isomers having the same MW [36].

EXPERIMENTAL

Potato tubers (*Solanum tuberosum*) of the middle-early-ripe cv Clivia, harvested in 1976, were purchased from 'Pommersche Saatzucht', Mainz and stored at 5° in the dark until used. Purified LDH iso 5 from rabbit and from pig muscle and LDH iso 1 from pig heart as well as glucose-6-phosphate dehydrogenase from yeast were from Boehringer, Mannheim. Ion exchanger (Servacel, 23 SS, p.a., kap. 0.74) was obtained from Serva, Heidelberg. Blue Sepharose CL-6 was a product of Pharmacia, Sweden.

Protein measurement. Protein was determined by the spectrophotometric method of ref. [37] using the following formula: mg protein/ml = $(A_{280} \cdot 2.067 - A_{260}) \cdot 1.155$ which was calculated taking BSA as standard.

Enzyme assay. Enzyme activities were measured by the change in A for NADH at 334 nm using a reaction mixture of 0.5 ml enzyme soln and 0.5 ml substrate soln. The substrate soln contained: 100 mM Tris–HCl buffer, 12.50 mM pyruvate and 0.125 mM NADH at pH 7.

Enzyme purification and extraction of soluble protein. All steps were carried out at *ca* 4°. After washing and peeling, 1 part (wt) of potatoes was homogenized under N_2 for 3 min in a homogenizer with one part (vol.) of extraction medium. The medium was slightly modified according to ref. [13] and contained: 100 mM Tris–acetate buffer, 1 mM EDTA·Na_2, 10 mM 2-mercaptoethanol and 0.1% (v/v) Triton X-100 at pH 9.5. The

resulting suspension was filtered through Miracloth and the filtrate centrifuged at 5500 *g* for 15 min. The supernatant was again centrifuged at 50000 *g* for 30 min and the clear supernatant taken as crude extract.

Desalting of crude extract and acid precipitation. Crude extract (300 ml) was desalted on a column of 1.4 l Sephadex G-25 c (1 × 35 cm). The gel was equilibrated and eluted with a 10 mM Tris–acetate buffer pH 8.5. The resulting protein soln was acidified with HOAc to pH 4.6–4.8 and precipitated protein removed by centrifugation (50000 *g* for 15 min). The supernatant which still contained LDH was brought to pH 8.5 with NaOH immediately after centrifugation.

DEAE chromatography. Desalted and acid precipitated protein extract (90 ml) were adsorbed to 12 ml of DEAE-cellulose previously equilibrated with 10 mM Tris acetate buffer pH 8.5. The ion exchanger was washed with equilibration buffer using a 10-fold vol. of the column vol. For further purification the exchanger was eluted with a gradient from 0 to 0.09 M KCl in 10 mM Tris acetate buffer pH 8.5. The total vol. of the gradient was the same as that of the washing buffer. After that the exchanger was washed with a vol. of half the column vol. of a 10 mM Tris–acetate buffer of pH 8.5 including 0.09 M KCl. Finally LDH was eluted with 0.25 M KCl in 10 mM Tris–acetate buffer pH 8.5. If no further purification was necessary a 0–0.4 M KCl gradient in 10 mM Tris acetate buffer was used to elute LDH.

Blue Sepharose chromatography. Dry Blue Sepharose powder (1 g) was swollen in H_2O and washed on a glass filter with 300 ml H_2O. The resulting affinity gel (3 ml) was packed in a column of 1.6 × 1.5 cm and equilibrated with a 10 mM Tris–acetate buffer pH 5. The purified soln of LDH was adjusted to pH 5 and adsorbed to the column. After extensive washing with the same buffer, LDH was either eluted with a pH-gradient or by the use of a 10 mM Tris–HCl buffer of pH 8. After extensive washing with this buffer and re-equilibration to pH 5 the column material was reused several times. For the determination of the capacity of the affinity gel, 0.5 ml of Blue Sepharose equilibrated in 10 mM Tris–acetate buffer of different pH values were thoroughly mixed with 0.5 ml LDH extract of a corresponding pH value. After 15 min at room temp., the suspension was centrifuged and the activity of the enzyme in the supernatant was compared with that of the starting enzyme.

Concentration and electrophoresis. During the purification procedure the protein was concentrated prior to electrophoresis according to the 'Centriflo' method. The final purification procedure was a large scale polyacrylamide gel electrophoresis [14], except that no large pore gel was used. Protein soln (1 ml) was separated on a polyacrylamide column (2.5 × 8 cm) at 40 mA.

SDS electrophoresis and thin layer gel chromatography. Purified isoenzyme 3 of potato tuber LDH was used to determine its subunit MW by SDS electrophoresis according to ref. [38]. The following proteins were used as markers: aldolase, glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase and egg albumin. Thin layer gel chromatography on Sephadex G-100 was used to determine the native MW of potato tuber LDH according to ref. [25]. The marker proteins were: chymotrypsinogen A, egg albumin, beef serum albumin, aldolase and catalase.

Determination of charge isomers. Charge and size isomers were determined according to the method of ref. [36]. Acrylamide concentrations of 6, 7, 8, 9 and 10% were used and the proportion of the acrylamide to N,N'-methylene-diacrylamide concentration was 30:1. The MW of the 3 plant LDH isoenzymes was also determined according to this method using glucose-6-phosphate dehydrogenase from yeast (MW 128 000) as a standard.

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