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AFFINITY PURIFICATION AND SUBUNIT STRUCTURE OF SOYA BEAN LACTATE DEHYDROGENASE

L. JERVIS, EWAN R. ROBERTSON and C. N. GODFREY SCHMIDT*

Department of Biology, Paisley College of Technology, High Street, Paisley, PA1 2BE, Renfrewshire, Scotland, U.K.

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Key Word Index—*Glycine max*; Leguminosae; lactate dehydrogenase; affinity chromatography; Blue Sepharose; ATP-Sepharose.

Abstract—The lactate dehydrogenase (LDH) from soya bean has been purified to homogeneity by affinity chromatography. The enzyme was purified by sequential adsorption onto Blue Sepharose and ATP-Sepharose followed by specific elution from each adsorbent by NADH. The enzyme preparations obtained by this double affinity purification had been purified over 17 000-fold and were homogeneous as judged by polyacrylamide gel electrophoresis (PAGE) under denaturing and non-denaturing conditions. The overall recovery of enzyme was high and the purification procedure was rapid. The enzyme is a tetramer with only one subunit type. In contrast to LDH from several other plant sources, no isoenzymes were detectable.

INTRODUCTION

The lactate dehydrogenases of animals and bacteria have been studied and reviewed in considerable detail [1, 2]. The enzyme from dogfish muscle has been completely sequenced and its three-dimensional structure has been elucidated [3, 4]. The co-factor binding site and the catalytic site have both been identified and the conformational changes occurring as a result of co-factor binding have been extensively studied. The NADH-binding site of animal lactate dehydrogenases consists of a super-secondary structure known as the dinucleotide fold. This structure, present in several other dehydrogenases [5, 6], appears to be a highly conserved region of protein structure and it has been used to deduce possible evolutionary relationships between dehydrogenases.

In contrast to the information available about animal lactate dehydrogenases, little detailed information exists about LDH from plant sources. The enzyme has been detected in several plants [7–13] and purified from a few of these [11–21]. The most highly purified preparation has been obtained by Poerio and Davies [13] from potato and they have carried out the most detailed kinetic and structural investigations on a plant LDH. Although other workers have carried out kinetic and even structural studies on LDH from potato [20, 22] and soya beans [14–18] their enzyme preparations have had low specific activities even though their preparations were claimed to be electrophoretically homogeneous.

One of the reasons for the lack of information and sometimes conflicting data concerning plant LDH lies in the low levels of this enzyme present in plants, a problem that compounds the difficulties of obtaining large amounts of pure enzyme. Recently, attempts have been made to overcome this problem by using affinity chromatographic techniques to develop highly specific and high yielding purification procedures. Both potato LDH [20] and soya bean LDH [16, 17] have been purified by non-specific elution from either Blue Sepharose or Blue Dextran Sepharose. However, the non-specific desorption procedures used resulted in relatively poor purifications of 2-fold [20] and 16-fold [16, 17] for the potato and soya bean enzymes, respectively. Considerably better degrees of purification have been achieved by genuine affinity chromatography of plant LDH on 5′-AMP-Sepharose (42-fold) [18], ATP-Sepharose (358-fold) [21], and oxamate-Sepharose (1284-fold) [13]. The latter adsorbent yielded homogeneous enzyme of very high specific activity.

In view of the increasing interest in plant lactate dehydrogenases we have developed a rapid, high-yielding affinity purification procedure for soya bean LDH. The procedure is based on the affinity of this enzyme for Blue Sepharose and ATP-Sepharose, these two adsorbents being used in series. Although the procedure was developed for soya bean LDH, it has been used successfully to purify LDH from several other plant sources to homogeneity.

RESULTS AND DISCUSSION

Chromatography on Blue Sepharose

Soya bean LDH has been partially purified by chromatography on Blue Dextran Sepharose [16, 17]. The enzyme was reported to bind tightly to this adsorbent and a high concentration of KCl was required to elute the enzyme; NADH failed to desorb the enzyme. Potato LDH has also been partially purified on Blue Sepharose [20]. NADH again failed to desorb the enzyme, a pH shift from 4.7 to 9.0 being required to effect elution. The non-specific elution procedures used resulted in relatively poor purification of the two enzymes.

We determined the effect of pH on the adsorption of soya bean LDH onto Blue Sepharose and also on desorption of the enzyme by NADH. Both adsorption and desorption phases of the chromatographic process

* Present address: Department of Botany, Rothamsted Experimental Station, Harpenden, Hertfordshire, U.K.

were quite sensitive to pH (Fig. 1a). Although adsorption of the enzyme to Blue Sepharose occurs over a wide pH range, desorption by NADH is only possible over a narrow pH range. However, when the chromatographic process is carried out at a suitable pH, not only is the enzyme adsorbed in a satisfactory manner, it can be eluted with NADH in high yield. The specific elution thus achieved brings about a purification of over 1000-fold (Table 1). The resulting enzyme preparation, although having a higher specific activity than any previously reported for soya bean LDH [14–18], is not homogeneous, there being at least five proteins present in addition to LDH (Fig. 2). Enzyme assays and activity stains of polyacrylamide gels indicated that both ADH and MDH were present in the LDH fraction but other proteins have not yet been identified.

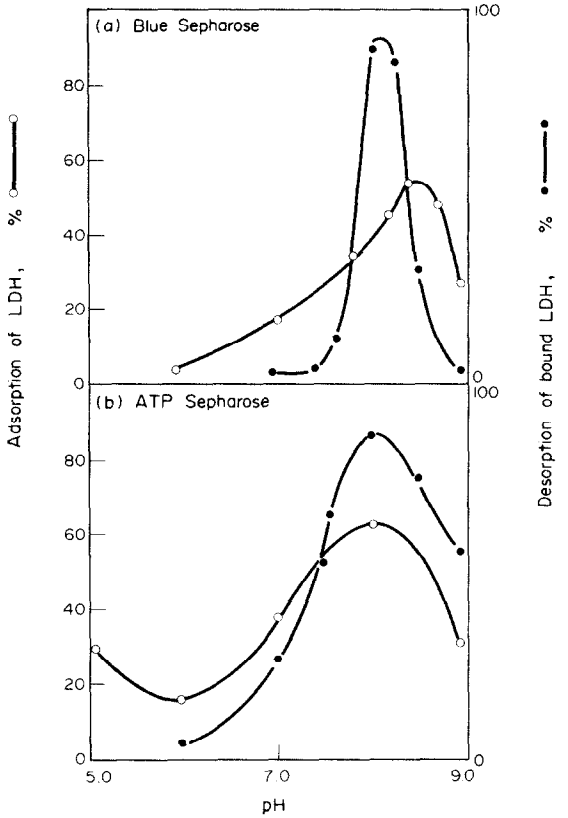

Fig. 1. Effect of pH on the adsorption and desorption phases of affinity chromatography of soya bean LDH. The effect of pH on the adsorption and desorption of soya bean LDH was determined under batch conditions (see Experimental). Adsorption is expressed as a percentage of the initial LDH level in a control vial (no adsorbent) removed from solution in 30 min. Desorption is expressed as a percentage of the amount of enzyme adsorbed that was released by 1 mM NADH in 30 min.

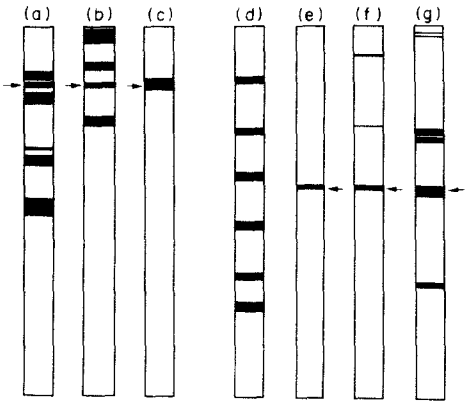

Fig. 2. Polyacrylamide gel electrophoresis of soya bean LDH at various stages of purification. (a) NADH-eluted material from Blue Sepharose to which 30–40% $(NH_4)_2SO_4$ fraction had been applied; (b) NADH-eluted material from ATP-Sepharose to which 30–40% $(NH_4)_2SO_4$ fraction had been applied; (c) material purified by passage through Blue Sepharose and ATP-Sepharose columns. All gels stained with Coomassie Blue G250. SDS-polyacrylamide gels: (d) Low MW marker proteins; (e) denatured soya bean LDH; (f) amidine cross-linked and denatured soya bean LDH; (g) high MW marker proteins plus denatured soya bean LDH. Low MW markers: Phosphorylase b (94000), albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20100), α-lactalbumin (14400). High MW markers: thryoglobulin (330000), ferritin (220000), catalase (60000), beef heart LDH (36000), albumin (67000). MWs given are subunit MWs.

Chromatography on ATP-Sepharose

The LDH from potato has been partially purified on ATP-Sepharose [21]. This adsorbent utilizes the affinity of potato LDH for ATP [12]. Soya bean LDH was also found to be susceptible to apparent allosteric inhibition by ATP and was found to bind to ATP-Sepharose from which it could be eluted by NADH. In contrast to potato LDH which binds to ATP-Sepharose only at pH values below 6.5, reflecting the pH-dependence of ATP inhibition on that enzyme [12], the soya bean enzyme binds to ATP-Sepharose over a wide pH range.

Elution of the enzyme by NADH is affected only slightly by pH (Fig. 1b).

Purification procedure for LDH

Examination of polyacrylamide gels of NADH-eluted material from Blue Sepharose and ATP-Sepharose

Table 1. Purification of soya bean LDH

Stage	Protein (mg)	LDH (I.U.)	Sp. act. (I.U./mg)	Purification	Yield (%)
Crude	43460	521	0.0119	1	100
Salt fraction	12690	495	0.039	3.27	95
Blue Sepharose	7.42	349	47.0	3949	67
ATP-Sepharose	1.38	292	211	17731	56

columns to which the 30–40% ammonium sulphate fraction of soya bean extract had been applied revealed that the two adsorbents had quite different specificities, apart from their joint affinities for LDH (Fig. 2). This result suggested that sequential use of these two adsorbents should yield homogeneous preparations of soya bean LDH. When the NADH-eluted material from a Blue Sepharose column was desalted to remove NADH and then applied to an ATP-Sepharose column, most of the protein passed through the latter column unretarded. Elution of the ATP-Sepharose column with NADH released the LDH which, after concentration by ultrafiltration, was found to be homogeneous (Fig. 2) and free from both ADH and MDH activities.

The purification procedure developed for soya bean LDH is summarized in Table 1. The final enzyme preparation was purified over 17 000-fold with a yield of 56%. The procedure is rapid and can be used routinely to prepare large amounts of pure LDH. In addition to soya bean LDH, the procedure has been used, with only slight modifications, to purify the LDH from several other plant sources including potato, turnip and rubber latex serum (Table 2).

Subunit structure

Recently, the subunit structure of potato LDH has been determined by the use of PAGE under denaturing conditions, and by the use of dimethyl suberimidate to covalently cross-link the subunits of the native enzyme [13, 20]. This enzyme exists in a number of isoenzymic forms, some of which can be shown to be due to proteolytic degradation [13]. Rothe *et al.* [20] suggested that these isoenzymes were size isomers with MWs of 115 000, 106 000 and 84 500, and that the native isoenzymes were generated from two subunit types having MWs of 41 000 and 43 000. Given the latter values, it is difficult to devise logical subunit compositions for the three isoenzymes. Poerio and Davies [13] also observed three isoenzymes in highly purified potato LDH preparations. All three isoenzymes had MWs of 150 000 and only one subunit type, of MW 37 500, was detected indicating a tetrameric structure for each isoenzyme. Poerio and Davies used homogeneous potato LDH for their subunit studies and avoided the action of protease by inclusion of PMSF in all buffers during the isolation procedure. Rothe *et al.*, on the other hand, used enzyme of low specific activity and did not use PMSF. In view of the conflict between these two sets of data it must be considered that the results presented by Rothe *et al.* [20] may be a reflection of serious proteolytic action on the enzyme during isolation resulting in the generation of isoenzymic forms of low catalytic activity and variable MW.

Studies on the subunit structure of soya bean LDH have also been carried out using SDS-PAGE [15]. The enzyme was found to have a MW of 140 000 with a subunit weight of 36 000. No evidence was found for the existence of isoenzymes or multiple subunit types. However, these studies were carried out on enzyme having a low specific activity (2.64 I.U./mg). Although the enzyme was reported to be electrophoretically homogeneous, it was purified by preparative electrophoresis and was, therefore, likely to appear homogeneous on analytical PAGE. Our preparation of soya bean LDH indicates that the enzyme preparation used by Barthova *et al.* for subunit structure determination was no more than 1–2% pure LDH. Consequently, we have re-assessed the subunit structure of this enzyme using homogeneous preparations.

Electrophoresis of purified soya bean LDH at several pH values and in a range of gel concentrations failed to reveal any evidence for isoenzymic forms of the enzyme (Fig. 3). Determination of the MW of the native enzyme by gel filtration [34] gave a value of 144 000. SDS-PAGE revealed only one protein band with MW of 38 000. The subunit could be resolved from the subunit of beef heart (H_4) LDH during co-electrophoresis (Fig. 2). Cross-linking of the native enzyme with dimethyl suberimidate followed by denaturation and SDS-PAGE gave the tetramer as the major cross-linked product (Fig. 2). These results indicate that soya bean LDH resembles both potato and vertebrate LDH in that it is a tetrametric enzyme. The reasons for the lack of isoenzymes of soya bean LDH are not clear. Even when enzyme was prepared in the absence of PMSF, only one form of the enzyme was present. The high level of endogenous protease inhibitor in soya beans may account for the lack of isoenzymes if the latter are due,

Table 2. Purification of plant LDH

Tissue	Stage	Sp. act. (I.U./mg)	Purification	Yield (%)
Potato	Crude	0.02	1	100
(Maris Piper)	25–50% $(NH_4)_2SO_4$	0.061	3	84
	Blue Sepharose	7.2	340	64
	ATP-Sepharose	228	11 400	57
Turnip	Crude	0.015	1	100
	25–50% $(NH_4)_2SO_4$	0.012	0.8	92
	Blue Sepharose	52	3466	81
	ATP-Sepharose	212	14 130	64
Rubber latex	Serum suspension	0.42	1	100
(freeze-dried serum)	Blue Sepharose	55	130	79
	ATP-Sepharose	225	535	53

Purification of LDH from several plants was carried out using slight modification of the procedure described for soya bean LDH. Potato: the ATP-Sepharose step was carried out in 50 mM KPi buffer, pH 6.0, as the enzyme is not adsorbed onto this adsorbent at pH 8.0; Rubber latex serum: the $(NH_4)_2SO_4$ fractionation stage was omitted.

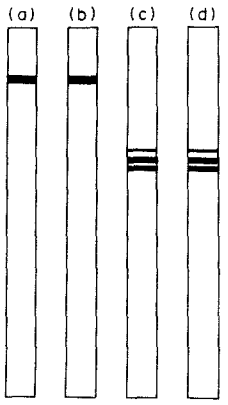

Fig. 3. Polyacrylamide gel electrophoresis of native soya bean and potato LDH. (a) and (c), soya bean LDH and potato LDH respectively stained for protein; (b) and (d), soya bean LDH and potato LDH respectively stained for LDH activity.

in plants, to the action of proteolytic enzymes. However, the lack of isoenzymes makes soya bean LDH a particularly suitable plant LDH for detailed structural studies.

EXPERIMENTAL

Materials. Blue Dextran 2000 and Sephadex G25 were obtained from Pharmacia (Uppsala, Sweden). Cibacron Blue 3GA was purchased from Ciba Geigy Ltd (Manchester, U.K.). Soya beans (*Glycine max* var. Fiskeby V) were from Thompson and Morgan Ltd (Ipswich, U.K.).

Adsorbent synthesis. ATP-Sepharose was synthesized as described elsewhere [21]. Immobilized ligand content, as determined by phosphate analysis, was in the range 4–5 μmol ATP/g of sucked-dry gel. Blue Sepharose C1-6B was synthesized as described in ref. [23]. Ligand content, as determined by acid hydrolysis [24], was 3.5 μmol Cibacron Blue 3GA/g sucked-dry gel.

Enzyme extraction and purification. All operations were performed at 5° unless otherwise stated. LDH was extracted from soya beans and fractionated with $(NH_4)_2SO_4$ as described in ref. [18]. The 30–40% $(NH_4)_2SO_4$ ppt. was suspended in, and dialysed against, 40 mM Tris–acetate buffer, pH 8.0, containing 2 mM 2-mercaptoethanol and 2 mM EDTA. The dialysed material was centrifuged and the supernatant was used for further purification.

Affinity chromatography. Optimum conditions for adsorption and desorption of LDH were determined initially under batch conditions. Plastic vials (3.0 ml capacity) containing 100 mg of sucked-dry adsorbent and 2.0 ml of buffered enzyme were tumbled end-over-end for 30 min at 5°. The adsorbents were then allowed to settle and 0.1-ml aliquots of the supernatants were assayed for unbound enzyme. To those vials in which adsorption had occurred was added 0.1 ml of 20 mM NADH. The vials were then tumbled again as above and, after settling, 0.1-ml aliquots of the supernatants were assayed for LDH.

Blue Sepharose chromatography. Columns (5 cm × 15 cm) were packed with adsorbent and equilibrated by washing with at least 5 columns vols of 40 mM Tris–acetate buffer, pH 8.0, containing 2 mM EDTA and 2 mM 2-mercaptoethanol. Enzyme samples prepared for chromatography as above were applied to the columns at a flow rate of 100 ml/hr at 5°. Columns were then washed with the above buffer until the effluent was free from protein. LDH was then eluted specifically by the addition of 1 mM NADH to the irrigating buffer.

ATP-Sepharose chromatography. The enzyme fractions eluted from Blue Sepharose by NADH were freed from NADH and buffer exchanged by gel filtration through a Sephadex G25 column (2.5 cm × 30 cm) equilibrated and eluted with the appropriate buffer (see text). The enzyme solutions were then applied to columns (1.5 cm × 10 cm) of ATP-Sepharose. After application of the enzyme, the columns were washed with starting buffer until the effluent was free from protein. LDH was then eluted from the adsorbent by addition of 1 mM NADH to the starting buffer. The eluted enzyme was freed from NADH by gel filtration as above and then concd by ultra-filtration. Glycerol was added to the final enzyme preparations to a final concn of 50% (v/v) and the preparations were stored at −20°.

Enzyme assays. LDH was assayed spectrophotometrically at pH 7.5 in 100 mM MES–Tris buffer containing 0.1% (w/v) bovine serum albumin at 25°. The assays were performed in the direction of pyruvate reduction using the substrate and co-factor concentrations described elsewhere [12]. Alcohol dehydrogenase (ADH) and malate dehydrogenase (MDH) were assayed as described elsewhere [25, 26]. One enzyme unit is defined as the amount of enzyme catalysing the oxidation of 1 μmol NADH/min under the conditions of assay. The inhibition of LDH by ATP was studied by the method of ref. [27].

Protein measurement. Protein was measured by the method of ref. [28] using bovine serum albumin as the standard protein.

Polyacrylamide gel electrophoresis. PAGE under non-denaturing conditions was performed as described in ref. [29]. Gels were stained for protein using Coomassie Brilliant Blue G250 as described in ref. [30]. LDH and MDH activities were detected in gels as described in ref. [31]. ADH was detected as described in ref. [32].

SDS-PAGE was performed as described in ref. [33].

MW determination. The MW of the native enzyme was determined by gel filtration [34].

Subunit cross-linking. Amidine cross-linking of soya bean LDH subunits was performed as described in ref. [13] using dimethyl subermidate.

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