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THE PURIFICATION OF PORCINE HEART LACTATE DEHYDROGENASE BY AFFINITY CHROMATOGRAPHY

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SUMMARY

The large scale purification of the heart (H_4) isoenzyme of porcine lactate dehydrogenase (EC 1.1.1.27) is described, utilizing an oxamate inhibitor affinity column in conjunction with conventional ion-exchange chromatography. The isoenzyme is obtained in relatively good (>60%) yield, and shows one band on electrophoresis.

INTRODUCTION

Affinity chromatography has been shown to be a very powerful technique for the purification of biological macromolecules. The techniques involved have been reviewed in detail elsewhere [1]. Recently O'Carra and Barry [2] using a minicolumn reported an affinity resin for lactate dehydrogenase (EC 1.1.1.27) based on the formation of the ternary complex of this enzyme with NADH and immobilized oxamate. The X_4 isoenzyme [3] of lactate dehydrogenase appears not to bind to this column, while the muscle (M_4) isoenzyme binds poorly (White, J. personal communication). Furthermore, it has been [3] reported that the coupling of potassium oxalate to aminohexylsepharose could not be achieved using the method reported by O'Carra and Barry [2]. We wish to report in this paper a procedure for the purification of large quantities of the heart (H_4) isoenzyme of porcine lactate dehydrogenase which allows for the isolation of active, electrophoretically pure, material based on the use of the oxamate inhibitor column prepared using the original method [2] and conventional DEAE-Sephadex chromatography.

EXPERIMENTAL

Materials

1,6-Hexanediamine was obtained from the Eastman Kodak Company, and used without further purification. Nicotinamide adenine dinucleotide was obtained from P-L Biochemical Ltd., Sepharose 4B from Pharmacia Fine Calbiochem., Inc. and 1-ethyl-3-(-3-dimethylaminopropropyl)-carbodiimide from Calbiochem, Inc. All were stored at 4 °C and used without further purification. Pork hearts were obtained fresh from Stark and Wetzel, Inc., and frozen at −15 °C until used.

Assay

The assay for lactate dehydrogenase activity was based on the reduction of pyruvate to lactate. The rate of reaction was followed by monitoring the decrease in absorbance of NADH at 340 nm. The assay was performed at ambient temperature (approx. 22 °C), using an assay solution prepared by dissolving 1.7 mg of sodium pyruvate and 6 mg of NADH in 50 ml of sodium phosphate buffer (67 mM PO_4^{3-}) pH 7.2. The final concentrations of pyruvate and NADH are 0.31 and 0.15 mM respectively. In a typical measurement, 3 ml of assay solution were placed in a 1-cm-pathlength cuvette. Immediately 10 μl of diluted enzyme solution was added and the decrease in the 340 nm band was monitored. Protein concentration was determined using an extinction coefficient of 1.4 $mg \cdot ml^{-1} \cdot cm^{-1}$ for lactate dehydrogenase.

Enzyme purification

The preparation of the oxamate column has been described elsewhere [2].

All procedures were performed at 4 °C. Heart tissue (2 kg), which had been allowed to thaw overnight, was freed of fat and arterial fibers, ground in a commercial electric meat grinder and extracted for 1 h with 5 l of distilled water. The mixture was then centrifuged for 20 min and the supernatant filtered through glass wool. To the resulting clear red extract, 60 g of preswollen DEAE-cellulose (Whatman DE-52) were added. The mixture was allowed to stand, with occasional stirring, for 20 min. The supernatant was decanted. The resin was washed with 0.02 M sodium phosphate buffer, pH 7.1, and then extracted for 20 min with the same buffer containing 0.5 M NaCl. The resin was removed by centrifugation, and solid NADH was added to the supernatant to give a final concentration of 0.2 mM. The solution was spun at 23 000 $\times$ *g* for 20 min to remove any particulate matter, and then applied to an oxamate column (600 ml), which had been equilibrated with 0.02 M sodium phosphate buffer, pH 7.1, containing 0.5 M NaCl and 0.2 mM NADH. The column was then washed with this buffer until the absorbance at 280 nm was close to zero. The reduced coenzyme was next removed from the buffer, and elution continued until activity was detected in the effluent. Elution was then halted, and the column was permitted to stand for 12 h to allow for dissociation of the ternary complex. Elution was then continued, and the active fractions combined and concentrated by pressure dialysis. The enzyme solution was dialyzed against 0.05 M sodium phosphate buffer, pH 7.2, for 48 h. The solution was then subject to ion-exchange chromatography using DEAE-Sephadex (A-50) and a 0.0–0.5 M NaCl gradient. The active fractions, containing a total of about 300 mg of pure H_4 lactate dehydrogenase, were combined and stored as a 75% saturation $(NH_4)_2SO_4$ slurry.

RESULTS

The results of the purification procedure are summarized in Table I.

The oxamate affinity column, as shown in Fig. 1, binds lactate dehydrogenase in the presence of reduced coenzyme. There is a lag of several column volumes between the removal of coenzyme from the eluting buffer and the dissociation of lactate dehydrogenase from the column. The enzyme is eluted as a very dilute solution. However, allowing the column to stand undisturbed for about 12 h after detection of activity in the effluent raises the subsequent enzyme concentration to approximately

TABLE I

SUMMARY OF THE RESULTS OF THE PURIFICATION

Procedure	Spec. act. (μmoles/min per mg)	Yield (%)	(×) fold purification
Crude extract	3.7	100	–
DEAE batch step	40.0	82 (82)*	10.8 (10.8)*
Oxamate column	193	74.6 (91)	52.9 (4.9)
DEAE column	270	63.4 (85)	74.1 (1.4)

* Numbers in parenthesis denote values for the individual procedures.

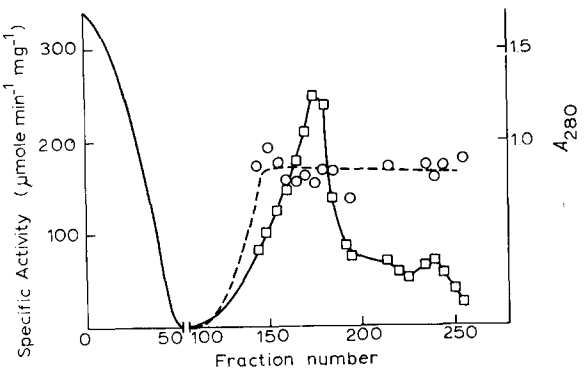

Fig. 1. Elution profile of the oxamate affinity column. (– – –) signifies specific activity and (———) signifies absorbance at 280 nm. NADH was removed after fraction 50.

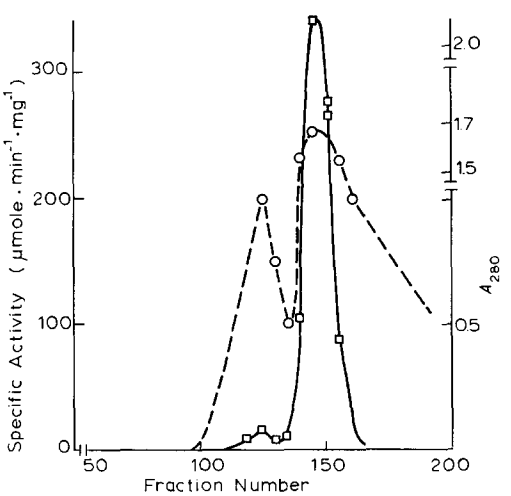

Fig. 2. Elution of lactate dehydrogenase from the DEAE-Sephadex column. (– – –) signifies specific activity and (———) signifies absorbance at 280 nm.

0.7 mg/ml. Electrophoresis of the enzyme, after concentration, using cellulose acetate strips, showed only bands which were distributed as might be expected for lactate dehydrogenase isoenzymes. Apart from the H_4 isoenzyme the other isoenzyme bands are very weak. The capacity of the column has been found to be on the order of 0.5 mg of lactate dehydrogenase per ml of column.

Ion-exchange chromatography on DEAE-Sephadex was employed to separate the lactate dehydrogenase isoenzymes. As shown in Fig. 2, two active fractions were observed on elution of the lactate dehydrogenase with a salt gradient. From their positions in the elution profile, and the absorbance maxima at 280 nm, the small peak is assigned to the H_3M isoenzyme, and the major peak to the H_4 isoenzyme. The H_4 isoenzyme obtained shows one band on electrophoresis, using cellulose acetate strips. Polyacrylamide gel electrophoresis indicates that the purity is greater then 99%. Furthermore, single crystals of the isoenzyme suitable for high resolution X-ray diffraction analysis have been grown successfully from this preparation, providing a further proof of purity. The specific activity of the pure isoenzyme is 270 μmoles/mg per min.

DISCUSSION

As stated previously [2], the binding of lactate dehydrogenase to the oxamate affinity column, is dependent upon the formation of the ternary complex lactate dehydrogenase–NADH–oxamate. The binding is highly specific, and there appears to be very little competition from other molecules present in the initial solution. Indeed, there is no significant change in column capacity or yield on eliminating the initial DEAE ion-exchange step, and applying crude extract to the column. The main reasons for the inclusion of the initial purification are to reduce the solution volume and avoid clogging of the column. The principle difficulty is the diluteness with which the enzyme is eluted from the column. Since the rate of dissociation of the ternary complex is rate limiting in the dissociation of bound lactate dehydrogenase, and furthermore since the rate of dissociation of the lactate dehydrogenase–NADH–oxamate ternary complex is slow [4], this result is not surprising. Indeed, allowing the column to stand undisturbed, helps to alleviate this problem.

The maximum capacity of the column has been found to be 0.5 mg of lactate dehydrogenase per ml of column. This is dependent on the dissociation constant of the ternary complex formed. Since the dissociation constant of the ternary complex increases on going from the heart (H_4) isoenzyme, to the muscle (M_4) isoenzyme [5], it seems reasonable to expect that the capacity (affinity) of the column for the M_4 isoenzyme may be lower than this value. The capacity implies that a relatively large column must be used to purify a large amount of M_4 lactate dehydrogenase.

The failure of the coupling reaction between potassium oxalate and aminohexylsepharose reported by Spielman et al. [3] may have been due to the addition of carbodiimide to the potassium oxalate solution in the absence of the resin. Under these conditions the decomposition of the oxalate occurs, however, it is apparent from the preceeding results that in the presence of aminohexylsepharose, the coupling reaction does take place.

Affinity chromatography, in conjunction with conventional ion-exchange chromatography, provides a direct method for the purification of the H_4 isoenzyme

of lactate dehydrogenase. However, when modifying this method for the purification of other lactate dehydrogenase isoenzymes, the factors mentioned above should be taken into consideration.

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