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# Purification of lipoamide dehydrogenase by affinity chromatography on propyllipoamide–glass columns

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SUMMARY

Lipoamide dehydrogenase (EC 1.6.4.3) from pig heart, yeast and *Escherichia coli* has been purified by chromatography on columns of propyllipoamide–glass beads. Chromatography of crude or partially purified lipoamide dehydrogenase on these columns has effected up to 100-fold purification of the enzyme in less than 30 min.

A variety of enzymes have been purified by affinity chromatography using substrates or pseudo-substrates bound to insoluble matrices[1-3]. The matrices have been predominantly compressible materials, such as cellulose or polydextrin, which produce easily compressible columns with relatively slow flow rates. We have purified lipoamide dehydrogenase (EC 1.6.4.3) using propyllipoamide covalently bound to an incompressible matrix of porous 96 % silica glass beads.

The column was prepared by refluxing the glass beads of 129 Å mean pore diameter (Corning Glass Works, Corning, N.Y.) with 10 % γ-aminopropyltriethoxysilane (Aldrich Chemical Co., Milwaukee, Wisc.) in toluene[4]. 1 g of the resulting alkylamine glass beads was shaken with 320 mg of lipoyl chloride in 3 ml of anhydrous dioxane for 2 h at 25 °C. The lipoyl chloride was prepared immediately prior to use by the action of oxalyl chloride (Aldrich Chemical Co., Milwaukee, Wisc.) on lipoic acid[5]. Approximately 2.19 mg of lipoic acid were bound per g of glass beads as determined by reducing the beads with $NaBH_4$ and then determining their sulfhydryl content with 5,5'-dithiobis (2-nitrobenzoic acid)

Other methods of preparing lipoylalkylamine glass were tried without much success. For example, activation of lipoic acid with dicyclohexylcarbodiimide to form lipoyl anhydride[6] and its subsequent reaction with alkylamine glass led to little incorporation of lipoic acid onto the glass bead matrix.

To prevent ion exchange by alkylamine groups which did not react with lipoyl chloride, the alkylamine glass beads were covered with acetic anhydride, and the acetic anhydride–glass mixture was warmed for 5 min on a steam bath. The acetylated beads were then washed three times with ethanol and twice with distilled water. If the derivatized glass beads are not pre-treated with acetic anhydride, the column functions as a weak ion exchanger.

Some purification of lipoamide dehydrogenase could be made solely on the basis of the ion-exchange property of alkylamine glass. This glass derivative may prove to be a very useful ion-exchange material. However, in our study, lipoamide dehydrogenase was only partially purified using such a column (or using non-acetylated propyllipoamide–glass), and the enzyme peak was electrophoretically heterogeneous, with numerous inactive protein contaminants.

For affinity chromatography of pig heart lipoamide dehydrogenase, the enzyme was first partially purified by Massey's procedure[7] through the first heat step. This prior

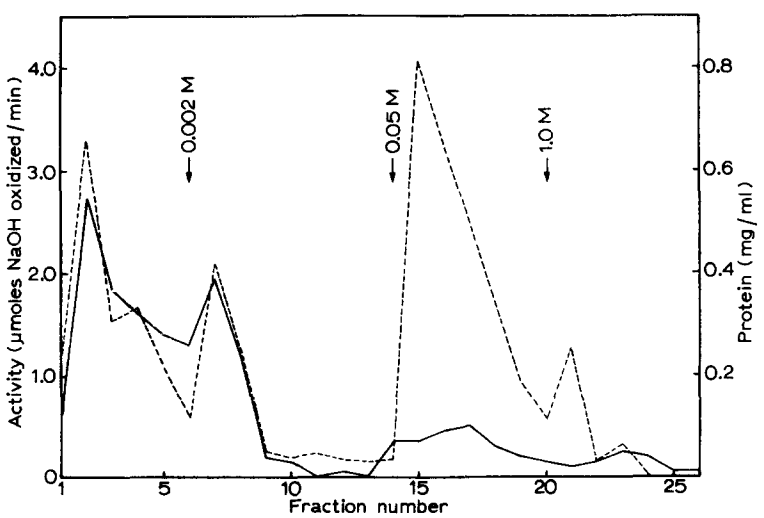


Fig. 1. Lipoamide dehydrogenase purification on propyllipoamide–glass. Pig heart lipoamide dehydrogenase (3.1 mg) purified through the first heat step of Massey[7] was applied to a 2.0 cm × 1.0 cm column of propyllipoamide–glass, 80–120 mesh. The crude enzyme had been exhaustively dialyzed against 0.002 M potassium phosphate buffer, pH 6.8 and the column was equilibrated with the same buffer prior to application of the enzyme solution. The column was washed with 10 column volumes of the 0.002 M buffer. After this, the enzyme was eluted with 0.05 M phosphate, pH 6.8 and finally with 1.0 M phosphate, pH 6.8. Each 1-ml fraction was assayed as described by Massey[7]. In this example, 89 % of the activity was retained on the column after sample application and approximately 60 % was eluted with the 0.05 M phosphate buffer, pH 6.8. A 67-fold purification was effected by collecting only the 0.05 M phosphate peak fractions. This peak was pure lipoamide dehydrogenase as judged by polyacrylamide gel electrophoresis. Repeated experiments show similar profiles. In no case was the entire enzyme activity retained on the affinity column.

purification served to eliminate lipoamidase from the extract. The latter enzyme catalyzes hydrolysis of lipoamide bonds and therefore would have caused degradation of the propyllipoamide–glass.

In a typical experiment, the heat-treated enzyme (3.1 mg) was exhaustively dialyzed against 0.002 M phosphate buffer, pH 6.5, containing 1 mM EDTA and then applied to a 2.0 cm × 1.2 cm column of *N*-propyllipoamide–glass. The column was then washed with the same buffer until no additional protein was removed. Normally, about 60 % of the enzyme was retained on the column and could be eluted with 0.05 M phosphate buffer, pH 6.5 (peak specific activity = 59 I.U./mg). In no case was all of the enzyme retained. The elution profile of one such preparation is shown in Fig. 1.

This procedure has yielded approximately 20–100-fold purification on several preparations. The amount of purification effected appears to depend upon the effectiveness of the prior heat treatment in removing inactive protein, for the enzyme in the 0.05 M phosphate peak fractions appeared to be homogeneous lipoamide dehydrogenase as judged by polyacrylamide gel electrophoresis.

Control experiments were similarly performed using columns of acetamide–glass beads. These were prepared by the same method used for preparing propyllipoamide–glass, with the sole change that treatment with lipoyl chloride was omitted. Lipoamide dehydrogenase was not retained on columns of acetamide–glass when applied and eluted under conditions identical to those described for purification of lipoamide dehydrogenase on propyllipoamide–glass beads.

Similarly, heat-treated extracts of *Saccharomyces cerevisiae* and *Escherichia coli* were purified on propyllipoamide–glass columns with results comparable to those described for pig heart lipoamide dehydrogenase.

The reported affinity of lipoamide dehydrogenase for its substrates ($K_m$ for lipoic acid, $2.0 \cdot 10^{-3}$ M at pH 5.9; $K_m$ for lipoamide, $5.0 \cdot 10^{-3}$ M at pH 6.5) [10] is lower than usually necessary for affinity chromatography[2]. However, the $K_m$ increases with increasing hydrophobicity of the side chain, *e.g.* lipoanilide has a higher $K_m$ than lipoamide[10] Therefore a column of the reduced substrate–glass bead complex was prepared. 20 ml of 10 % $NaBH_4$ in 0.1 M $Na_2CO_3$, pH 8.0, cooled to 4 °C, were passed through a 2.0 cm × 1.2 cm column of propyllipoamide–glass beads at a rate of 2 ml/ml. The reduced column was washed with 2 column volumes of 0.1 M phosphate buffer, pH 5.0 and then 100 column volumes of 0.002 M phosphate buffer, pH 7.2. When this propyldihydrolipoamide–glass was used as the column matrix, the results were identical with the purification as described using the oxidized form of the substrate bound to the glass matrix.

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