APPLICATION OF IMMOBILIZED ASPARTATE AMINOTRANSFERASE AND IMMOBILIZED ASPARTATE AMINOTRANSFERASE–MALATE DEHYDROGENASE COUPLED SYSTEM TO MICRO-ASSAY OF L-ASPARTIC ACID

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1. Introduction

Usefulness of immobilized enzymes in analyses has been well recognized [1,2]. Especially, immobilized multi-enzyme systems would be the most promising materials for micro-analyses of various biological compounds. Newirth et al. [3] have reported the determination of phosphoenolpyruvate by use of the combined system of immobilized pyruvate kinase and immobilized lactate dehydrogenase. Automatic determination of disaccharides has been performed by Inman and Hornby with some immobilized linked enzyme systems [4].

In previous papers [5,6], the present authors reported the preparation of immobilized tryptophanase and immobilized tryptophanase-lactate dehydrogenase coupled system and the application to the micro-assay of L-tryptophan.

This paper describes the immobilization of aspartate aminotransferase (AAT) and of a coupled system of AAT and malate dehydrogenase (MDH) and their application to a micro-assay of L-aspartate.

2. Materials and methods

2.1. *Materials*

Cytoplasmic aspartate aminotransferase (L-Aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1.) (AAT) from pig heart muscle and malate dehydrogenase (L-Malate:NAD oxidoreductase, EC 1.1.1.37) (MDH) from pig heart mitochondria were purchased from Boeringer Mannheim GmbH. Apoenzyme of AAT was prepared by the method of Scardi et al. [7]. Sepharose 4B, sodium salt of NADH, pyridoxal 5′-phosphate and other reagents used in this study were obtained from commercial sources.

2.2. *Enzyme assay*

It is well established that AAT catalyzes the stoichiometric conversion of L-aspartate to oxalacetate in the presence of excess 2-oxoglutarate. The amount of oxalacetate produced is assayed spectrophotometrically [8] or measured by following the absorbance change of NADH by coupling with MDH reaction [9, 10]. Hence, application of immobilized AAT or immobilized AAT–MDH coupled system can be expected to provide a convenient and economical method for micro-assay of L-aspartic acid. The specific activity of AAT preparation used for immobilization was 145 units/mg-protein (at 37°C) when assayed by the former method. The activity of MDH was assayed spectrophotometrically by using oxalacetate and NADH as substrates according to the description of Kitto [11]. The specific activity of MDH preparation used for immobilization was 540 units/mg-protein (at 37°C). Protein concentrations were determined by the method of Lowry et al. [12].

The activities of immobilized enzymes were assayed by a batch method as follows: the reaction mixture containing the immobilized enzyme was incubated with shaking in a glass funnel equipped with a sintered glassfilter for 15–60 sec. After the reaction was over,

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the reaction mixture was filtered and the amount of product or the decreased amount of substrate in the filtrate was determined. The comparison of activities of immobilized enzyme and the soluble counterpart was carried out by using an equal amount of protein.

2.3. *Immobilization of AAT*

AAT was immobilized covalently on CNBr-activated Sepharose 4B in a way similar to that described by Axén and Ernback [13]: activated Sepharose 4B (0.5 g, wet) was mixed with 1.0 mg of apo- or holo-AAT dissolved in 0.1 ml of 0.05 M Tris–HCl buffer, pH 7.0. The coupling reaction was allowed to proceed for 16 hr at 4°C. The immobilized enzyme thus prepared was throughly washed with 0.1 M potassium phosphate buffer of pH 8.5 and 5.5, alternately. The amount of enzyme bound to Sepharose was determined by the difference between the initial amount of enzyme and that of free enzyme remaining in the supernatant after the immobilization process.

The coupled immobilization of AAT and MDH on Sepharose 4B was carried out as follows: CNBr-activated Sepharose 4B (0.3 g, wet) was added to the enzyme solution containing 0.36 mg of holo-AAT and 0.2 mg of MDH in 0.6 ml of 0.05 M Tris–HCl buffer, pH 7.0. Then, the mixture was treated in an analogous manner as described above. The activity of AAT thus immobilized with MDH was assayed in three different manners as mentioned below.

2.4. *Determination of L-aspartate with immobilized AAT or AAT–MDH coupled system*

Determination of L-aspartate after conversion to oxalacetate using immobilized AAT was carried out as follows: Sepharose-bound AAT (0.7 ml) was placed into a small column (8.0 mm, diameter), jacketted for temperature control (37°C). After the column was washed throughly with 0.05 M Tris–HCl buffer (pH 8.0) containing 10 μM pyridoxal 5′-phosphate and 6.25 mM 2-oxoglutarate, 0.5 ml of the sample solution containing 0.05 to 1.00 μmole of L-aspartate in the same buffer solution was applied onto the column and allowed to stand for 5 min, and then the product was eluted from the column by washing with 1.5 ml of the same buffer. The increase in the absorbance at 280 nm in the eluate (2.0 ml) was determined and the amount of oxalacetate formed was calculated according to the procedure of Cohen [8].

Determination of L-aspartate by use of immobilized AAT–MDH coupled system was carried out as follows: the small column packed with Sepharose-bound AAT–MDH coupled system (0.7 ml) was washed throughly with a buffer solution consisting of 0.05 M potassium phosphate buffer (pH 7.5), 10 μM pyridoxal 5′-phosphate, 0.14 mM NADH and 20 mM 2-oxoglutarate. After the absorbance at 340 nm, due to NADH, of the washings reached a constant level, 0.5 ml of the sample solution containing 5–45 nmoles of L-aspartate in the same buffer was applied onto the column, kept for 5 min and then eluted with the above-mentioned buffer. The elution was continued until the absorbance owing to NADH (340 nm) of the eluate (each fraction, 0.5 ml) was restored to the initial constant level. The decrease in the absorbance at 340 nm from the initial level in each fraction was summed up and the total amount of NADH decreased was calculated. The molar extinction coefficient, 6.22×10^3 M^{-1} cm^{-1}, was used for NADH.

2.5 *Determination of the activities of AAT and MDH of the immobilized AAT–MDH coupled system*

To examine the respective activities of AAT and MDH in their immobilized coupled system, the following procedures were employed: 1) the AAT activity alone was expressed by the amount of oxalacetate formed from L-aspartate, when the reaction mixture free of NADH was applied onto the column of the coupled system, and 2) the MDH alone was expressed by the decreased amount of NADH, when oxalacetate and NADH were used as the substrate. Furthermore, the efficiency of the linked reactions converting L-aspartate to L-malic acid was compared in the presence and absence of free MDH, in order to examine whether the AAT–MDH coupled system would be well-balanced or not.

3. Results and discussion

Apo- or holo-AAT was immobilized on CNBr-activated Sepharose as mentioned above. In each case, approx. 60% of protein used was immobilized and the activity of immobilized enzyme was approx. 20% of that of the soluble enzyme used for the immobilization (table 1, expts. 1 and 2). With CNBr-activated Sepharose 4B, enzymes have been shown to be immobilized via amino group(s) on the surface of enzyme [14]. It has been

Table 1
Immobilization of Aspartate Aminotransferase (AAT) with or without Malate Dehydrogenase (MDH) on CNBr-activated Sepharose

Expt.	Total activity of enzyme used (units)		Efficiency of immobilization (%)	Total activity of immobilized enzyme (units)	Retained activity (%)
1	AAT (apo)	145	62	29.0	20.0
2	AAT (holo)	145	57	30.0	20.6
3	AAT (holo)	52.5	67	11.5	22.0
	+ MDH	108		28.9	26.8

In each case, the concentration of enzyme used was 1.0 mg/ml in 0.05 M Tris–HCl buffer, pH 7.0 and the amount of CNBr-activated Sepharose used was 0.5 g (wet)/ml-enzyme solution. Activity of AAT was assayed by measuring the amount of oxalacetate formed from L-aspartate. Efficiency of immobilization was expressed by the ratio of the amount of protein immobilized on Sepharose to the initial amount of enzyme protein used for immobilization. In expt. 3, the efficiency of immobilization was calculated without distinguishing two enzymes (AAT and MDH). Activity of MDH in the AAT–MDH coupled system was determined as mentioned in the text.

determined that cytoplasmic AAT from pig heart has 10 lysine residues per mole of one enzyme subunit (mol. wt. = 46 344) and one lysine residue (numbered 258), essential for the enzyme activity, is present at the active center [15]. The lysine residue at the active site is in the free state in apoenzyme, but is linked via aldimine linkage with pyridoxal 5′-phosphate in holoenzyme. If apo-AAT (dimer) would be immobilized on CNBr-activated Sepharose through this lysine located at the active site, the active center should become inactive. Consequently, the immobilized enzyme prepared from apoprotein, even after reconstituted with pyridoxal 5′-phosphate at another remaining active site, would show a fairly low activity as compared with that prepared from holoenzyme. The above-mentioned results, however, seem to indicate that the lysine residue at the active site of apo-AAT which should be buried within the ternary conformation of the enzyme molecule is not so reactive to the activated group(s) of Sepharose as other lysine residue(s) on the enzyme surface.

The immobilized AAT thus obtained was conveniently used for the assay of L-aspartic acid. Using the immobilized AAT column, 0.05–0.40 μmole of L-aspartic acid was converted quantitatively to oxalacetic acid, which was easily assayed by determining the absorbance at 280 nm (fig. 1). When AAT was immobilized together with MDH, the activity of AAT alone in the coupled system was measured by the amount of oxalacetate formed by using the reaction mixture free of NADH. The value, 11.5 units, was 22% of the soluble AAT used as the starting material (table 1, expt. 3). This result was almost identical with the case of immobilization of AAT alone (table 1, expt. 2). Furthermore, the immobilization efficiency of AAT together with MDH was almost similar to that of AAT alone. These results suggest that AAT and MDH have

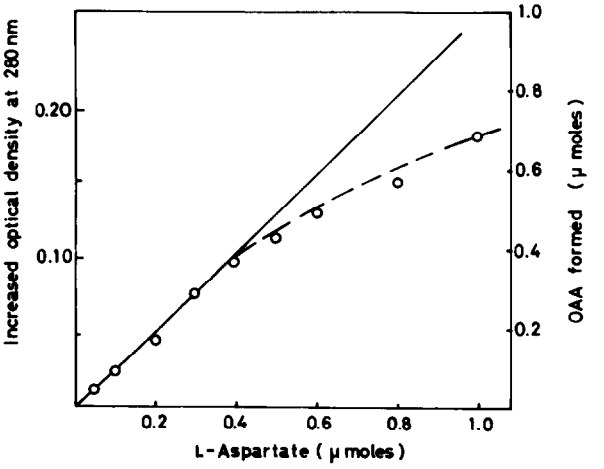

Fig. 1. Amounts of oxalacetate formed vs. graded amounts of L-aspartic acid applied to immobilized AAT column. The plots of observed values (o——o) deviated from the theoretical line. When the reaction time is elongated, the plots become linear.

substantially similar reactivities toward CNBr-activated Sepharose and that the conformation of AAT immobilized on Sepharose was not affected by the concomitant immobilization of MDH under the experimental conditions employed.

The comparison of the activities of immobilized AAT–MDH coupled system assayed in the presence and absence of free MDH indicated that the preparation tested was not necessarily well-balanced, that is, the amount of MDH in the immobilized coupled system was insufficient compared with the amount of AAT.

However, the immobilized AAT–MDH coupled system was found to be useful for the assay of a relatively small amount of L-aspartic acid. When L-aspartic acid ranging from 5 to 45 nmoles was applied to the column of immobilized coupled system, the acid in this range was able to be assayed conveniently and specifically by following the decrease in the absorbance of NADH as shown in fig. 2. The sensitivity for L-aspartic acid of the immobilized coupled system

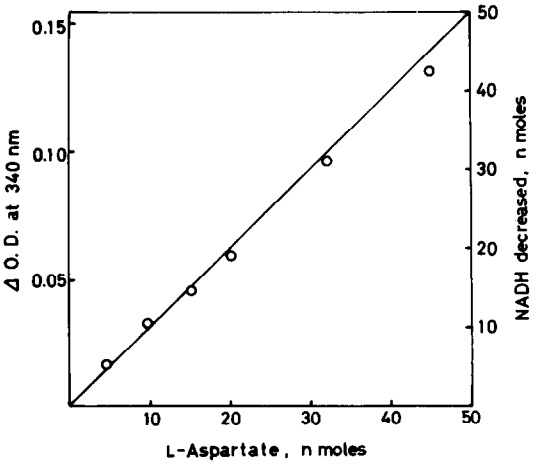

Fig. 2. Amounts of decreased NADH vs. graded amounts of L-aspartic acid applied to a column of immobilized AAT–MDH coupled system.

was superior by approx. 10-fold to the immobilized AAT alone.

These two enzyme systems retained their original activities after the continuous reactions during more than 24 hr at 37°C. These stabilities indicate the usefulness of the immobilized enzyme systems for continuous and automatic analysis of L-aspartic acid.

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