APPLICATION OF IMMOBILIZED TRYPTOPHANASE OR TRYPTOPHANASE–LACTATE DEHYDROGENASE COUPLED SYSTEM FOR ASSAY OF L-TRYPTOPHAN

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1. Introduction

The usefulness of immobilized enzymes in analysis has been recently recognized. Lactate dehydrogenase immobilized in polyacrylamide gel was successfully used for determination of lactate by Hicks et al. [1]. Katchalski et al. utilized immobilized urease for the assay of urea in urine and serum [2]. The advantages of these methods consist of the facts that immobilized enzymes can be used repeatedly or continuously and that the products of the enzyme reactions are easily separated from the enzyme catalysts. Furthermore, immobilized enzymes combined with appropriate analyzer(s) promise to extend their applications for reagentless and automatic analyses. For example, Updike et al. have combined an electrochemical procedure with immobilized glucose oxidase and successfully applied to an 'enzyme electrode' for assay of glucose [3]. The application of immobilized enzymes in flow microcalorimetry has been reported by Johansson et al. [4].

This communication deals with immobilization of tryptophanase and of tryptophanase–lactate dehydrogenase coupled system and their utilization for assay of L-tryptophan. The most common analytical procedure for L-tryptophan involves condensation with aldehyde, e.g., *p*-dimethylaminobenzaldehyde (PDAM), followed by oxidation with nitrite [5]. In comparison with tryptophan, however, indole is conveniently determined with only PDAM with much higher sensitivity [6]. Tryptophanase has been known to catalyze the stoichiometric conversion of tryptophan to indole, pyruvate and ammonia [7]. Hence, Watanabe and Snell determined a slight amount of tryptophan after converting it to indole with soluble tryptophanase [8]. Application of immobilized tryptophanase would provide a more convenient and economical method for assay of tryptophan. Since tryptophanase activity can be assayed spectrophotometrically by measuring pyruvate formation by use of a coupled system containing reduced nicotinamide-adenine dinucleotide and lactate dehydrogenase [9], a procedure for determination of tryptophan using immobilized a multienzyme system (tryptophanase and lactate dehydrogenase) is also examined.

2. Materials and methods

2.1. *Materials*

Crystalline lactate dehydrogenase from rabbit muscle (Sigma, Type I), sodium salt of NADH, pyridoxal 5'-phosphate and other reagents used in this study were obtained from commercial sources. Indole color reagent (acid–Ehrlich reagent) was prepared by mixing 5 volumes of 5% of *p*-dimethylaminobenzaldehyde in 95% ethanol with 12 volumes of 5% sulfuric acid in *n*-butanol.

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2.2. *Immobilized tryptophanase*

Tryptophanase was prepared according to the method of Kagamiyama et al. from *E. coli* B/1t 7-A [10]. This strain was kindly donated by Dr H. Wada and Dr Y. Morino, Osaka University School of Medicine. The specific activity of the enzyme used was about 4 enzyme units/mg protein. Although the coupling of tryptophanase to Sepharose-bound pyridoxal 5'-phosphate, followed by reduction with $NaBH_4$, yields the most active immobilized tryptophanase [11], this study employed direct binding of the enzyme to CNBr-activated Sepharose: Activated Sepharose 4B (10 ml) was mixed with 40 mg of tryptophanase dissolved in 5.0 ml of 0.1 M K-phosphate buffer (pH 7.0). The mixture was gently agitated for 24 hr at 4°C. By this treatment, about 80% of the initial amount of protein was immobilized on Sepharose. Immobilized tryptophanase thus obtained exhibited the specific activity of about 3.8 units/ml Sepharose. The immobilized enzyme preparation, when used in continuous enzyme reactions during 5 days at 37°C, showed only a slight decrease (approx. 3%) in the enzymatic activity after this period.

2.3. *Immobilization of tryptophanase and lactate dehydrogenase-coupled system*

Lactate dehydrogenase (5.0 mg, 75 units) and tryptophanase (5.0 mg, 20 units) were mixed in 0.1 ml of 0.1 M K-phosphate buffer, pH 7.0. Immobilization was carried out as described above with 2.5 ml of CNBr-activated Sepharose. About 60% of total protein used initially was immobilized. The immobilized enzyme preparation thus obtained exhibited both tryptophanase activity (0.80 units/ml Sepharose) and lactate dehydrogenase activity (6.4 units/ml Sepharose). The lactate dehydrogenase activity was assayed as described by Kornberg [12]. These activities of the immobilized multienzyme system were scarcely decreased after the continuous reactions during 3 days period at 37°C.

2.4. *Procedure*

Determination of L-tryptophan as indole using immobilized tryptophanase was carried out as follows: Sepharose-bound tryptophanase (2.5 ml) was placed into a small column (8.0 mm diameter), jacketted for temperature control (37°C). After the column was washed with 0.1 M K-phosphate buffer (pH 8.0) containing 40 μM pyridoxal 5'-phosphate, 0.5 ml of the sample solution containing 5 to 40 nmoles of tryptophan in the same buffer was kept in the column for 10 min and then eluted from the column by washing with 6 ml of the same buffer. A molar extinction coefficient, 5.25×10^3 M^{-1} cm^{-1} at 280.5 nm (in 0.1 N NaOH), was used for the estimation of tryptophan. Indole formed was extracted from the combined eluate with 3.0 ml of toluene and the color was developed by adding the equal volume of a freshly mixed acid–Ehrlich reagent. After 20 min, the color intensity was read at 570 nm.

Determination of L-tryptophan by use of immobilized tryptophanase and lactate dehydrogenase-coupled system was carried out as follows: Sepharose-bound multienzyme system (2.5 ml) was used as described above. The column was washed throughly with the elution buffer consisting of 0.1 M K-phosphate buffer (pH 8.0), 10 μM pyridoxal 5'-phosphate and 50 μM NADH. After the absorbance at 340 nm owing to NADH to eluate reached a constant level, 2.0 ml of the sample solution containing 5 to 40 nmoles of tryptophan in the same buffer was applied onto the column, kept for 10 min, and then eluted with the elution buffer. This elution procedure was continued until the absorbance at 340 nm of each eluate fraction (2 ml) was restored again to the initial constant level. The degree of 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. A molar extinction coefficient, 6.22×10^3 M^{-1} cm^{-1}, was used for NADH.

3. Results and discussion

The stability of the immobilized tryptophanase or immobilized tryptophanase and lactate dehydrogenase-coupled system during the continuous reaction as described in Methods satisfied initial requirements for the analytical application of these immobilized enzymes. As shown in fig. 1, the calibration curve of tryptophan treated with the immobilized tryptophanase column was almost identical with that of free indole, when acid–Ehrlich reagent was used. Since indole is more sensitive than tryptophan toward the reagent [6], and indole is formed specifically from L-tryptophan by tryptophanase reaction, the method using

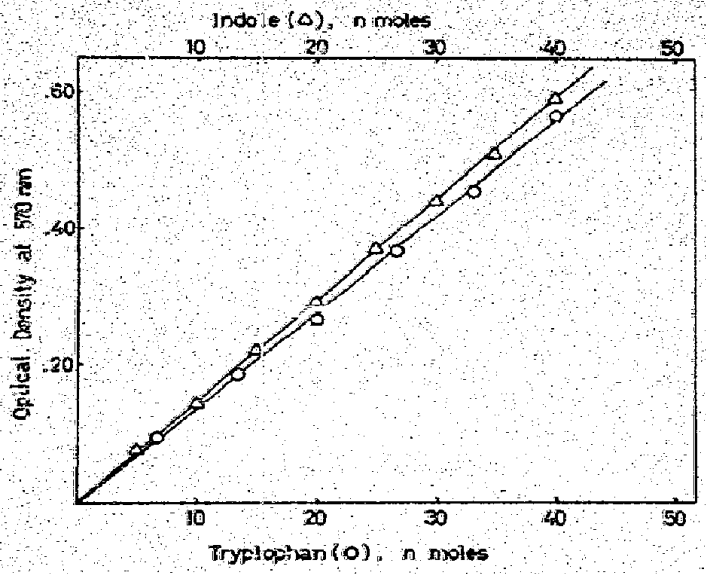

Fig. 1. Comparison of color formation in the Ehrlich reaction of free indole (△) and of tryptophan after conversion to indole with immobilized tryptophanase (○).

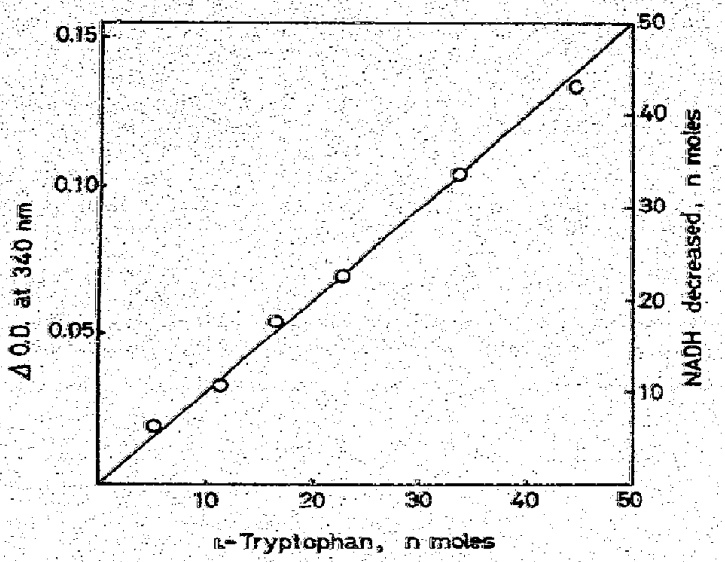

Fig. 2. Amounts of decreased NADH vs. graded amounts of tryptophan applied to immobilized tryptophanase and lactate dehydrogenase-coupled system.

immobilized tryptophanase is considered to be a specific as well as convenient assay method for L-tryptophan.

Moreover, the amount of tryptophan applied to a column of immobilized tryptophanase and lactate dehydrogenase-coupled system was almost quantitatively determined by the decrease of NADH as shown in fig. 2. Since this method does not require any special reagents except for the coenzymes for both enzymes, a reagentless as well as automatic analytical technique will be developed based on this principle. This kind of assay method will be extended for the automatic analysis of amino acids which can be converted to pyruvate by appropriate enzymes.

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References

[1] Hicks, G. P. and Updike, S. J. (1966) Anal. Chem. 38, 726–730.
[2] Riesel, E. and Katchalski, E. (1964) J. Biol. Chem. 239, 1521–1524.
[3] Updike, S. J. and Hicks, G. P. (1967) Nature 214, 986–988.
[4] Johansson, A., Lundberg, J., Mattiasson, B. and Mosbach, K. (1973) Biochim. Biophys. Acta 304, 217–221.
[5] Udenfriend, S. and Peterson, R. E. (1957) Methods in Enzymol. Vol. III, pp. 613–614.
[6] Kupfer, D. and Atkinson, D. (1964) Anal. Biochem. 8, 82–94.
[7] Wood, W. A., Gunsalus, I. C. and Umbreit, W. W. (1947) J. Biol. Chem. 170, 313–321.
[8] Watanabe, T. and Snell, E. E. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1086–1090.
[9] Morino, Y. and Snell, E. E. (1967) J. Biol. Chem. 242, 2793–2799.
[10] Kagamiyama, H., Wada, H., Matsubara, H. and Snell, E. E. (1972) J. Biol. Chem. 247, 1576–1586.
[11] Ikeda, S. and Fukui, S. (1973) Biochem. Biophys. Res. Commun. 52, 482–488.
[12] Kornberg, A. (1955) Methods in Enzymol. Vol. 1, pp. 441–443.