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Fiber optic biosensor of immobilized firefly luciferase

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Abstract: Luciferase from firefly lantern extract was immobilized on CNBr-activated Sepharose 4B. The kinetic properties of immobilized luciferase were extensively studied. The $K_m{}'$ for D-luciferin is 11.9 μmol/L, the optimum pH and temperature for Sepharose-bound enzyme were 7.8 and 25℃ respectively. A luminescence fiber optic biosensor, making use of immobilized crude luciferase, was developed for assay of ATP. The peak light intensity was linear with respect to ATP concentration in range of $10^{-9}-10^{-5}$ mol/L. A biological application was also demonstrated with the determination of serum ATP from rats bred in low versus normal oxygen environments.

Key words: Firefly luciferase, ATP, Immobilized enzyme, Fiber optic biosensor
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INTRODUCTION

One problem in analytical biochemistry is how to assay accurately and quickly the microquantities of ATP, a very important energy carrier in living organisms. Conventional ATP analytical methods are generally not particularly sensitive, specific or easy to handle. However, many of these problems could be solved by the use of the firefly luciferase ATP detection system (Lagido et al., 2001). The overall reactions were as follows (Leach, 1981):

$$LH_2 + ATP + E \rightarrow E\cdot LH_2\text{-}AMP + PPi \quad (1)$$

$$E\cdot LH_2\text{-}AMP + O_2 \rightarrow E\cdot P^* + CO_2 + AMP \quad (2)$$

$$E\cdot P^* \rightarrow E\cdot P + hr \quad (3)$$

(LH_2, D-luciferin; E, firefly luciferase; P^*, oxyluciferin; hr, light)

The applicability, however, is limited by the enzyme cost. In order to reduce its consumption, broaden its application and make the technique automatic and continuous, attempts had successively been made to immobilize firefly luciferase on different carriers since Lee et al. (1981) first immobilized the enzyme successfully. In these cases, expensive and pure luciferase was generally used. In some reports ATP was determined by immobilized enzyme put directly in the cuvette of a luminometer. In this application it is difficult to ensure the reaction homogeneous and to make the analysis automatic. Several novel types of fiber optic biosensor were recently reported (Vangelis et al., 2002; Tservistas et al., 2001; Maria et al., 2000; Michel et al., 1998; Blum et al., 1988). This paper describes the direct immobilization of crude firefly luciferase extract. A biosensor, made up of immobilized enzyme and an optical fiber, can perform continuous measurements of ATP. Preliminary application of the sensor in the analysis of actual blood samples is also described.

EXPERIMENTAL PROGRAM

1. Materials

Crude firefly luciferase (firefly lantern extract) and D-luciferin (LH_2) were purchased from Sigma; ATP was offered by Shanghai Institute of Biochemistry; Cyanogen bromide was from E. Merck; Dithiothreitol (DDT) was supplied by Serva; other chemicals were analytical grade. FG-300 luminometer was from Shanghai Institute of Plant Physiology; optical fiber (0.6

cm in diameter × 80 cm in length) was from Department of Electron Engineering, Dongnan University.

2. Methods

1) Enzyme immobilization

Sepharose 4B was activated by Cyanogen bromide according to the method of Ji Xinsong et al. (1984). The 1.5 g CNBr-activated Sepharose 4B was placed in 4ml 10 mg/ml of luciferase and stirred overnight at 4 ℃, then washed thoroughly with 0.1 mol/L phosphate buffer, pH 7.0 and kept at 4 ℃.

2) Assays of enzyme activity

The assays were performed by recording light emission as soon as the 0.5 ml 4 mmol/L ATP was rapidly injected with a syringe into a solution containing 1ml 0.02 mol/L Tris-acetate buffer (pH 7.8, containing 4 mmol/L EDTA), 0.2 ml luciferase solution (or immobilized enzyme suspension). The specific-enzyme activity was calculated according to the following formula:

Enzyme activity (U) = (dimension of the recorder (μV) / multiple in the luminometer) × 10 × height of light peak shown in recording paper (cm)

Where dimension and multiple are measuring range selection parameters of the recorder and the luminometer respectively.

The amount of enzyme resulting in a signal of 1μV in luminometer is defined as one unit of luciferase activity.

3) Fiber optic biosensor for ATP assays

(1) ATP assay

The construction of the fiber optic biosensor is shown in Fig. 1. One end of the optical fiber was connected to an opening at the upper center of a 1.5 ml reaction cell, and the other was fixed closely to the end of the photomultiplier tube in the luminometer. Immobilized enzyme was put into the reaction cell before assay. After the 0.05 mol/L Tris-acetate buffer, pH 7.8, containing 1mmol/L EDTA, 10 mmol/L $MgSO_4$ was pumped into the reaction cell of the biosensor and 10 μL 500 mg/ml LH_2 was injected into the cell, make sure that the background luminescence was recorded simultaneously. The light intensity emitted upon injection of 50 μL ATP sample into the mixture was recorded. The cell was washed with the buffer for 10 minutes before the next assay.

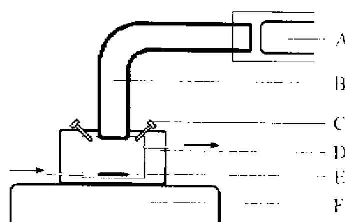

Fig. 1 Design of the fiber optic biosensor

A: photomultiplier tube; B: optic fiber; C: injection hole; D: lightproof reaction cell; E: stirring bar; F: magnetic stirrer

(2) Preparation of blood serum of rat

At set time intervals, blood samples were taken by the cut neck method from SD rats, which were continuously bred in a low oxygen cabinet simulating 5 000 meters above sea level environment and pretreated according to the method (Neuberg, 1944) described previously.

RESULTS AND DISCUSSION

1. Catalytic properties of immobilized luciferase

The activity of CNBr-activated Sepharose bound firefly luciferase was up to about 750 U/g carrier, the activities recovered on Sepharose were over 18%. The properties of immobilized enzyme were as follows:

1) The thermostability of immobilized luciferase

Sepharose bound firefly luciferase suspended in phosphate buffer in the presence and in the absence of 4 mmol/L DTT, and soluble enzyme were incubated simultaneously at 30 ℃ to compare their thermostability. The results are shown in Fig. 2. Immobilized luciferase was more stable than the soluble enzyme. Also the stability of immobilized enzyme was further improved when it was stored in the presence of DTT. This suggested that immobilized luciferase had active SH groups and was more stable when stored in solution with an SH group protection such as DTT.

2) K_m' for LH_2 of luciferase

The apparent K_m (K_m') values for substrate LH_2 of Sepharose bound and soluble crude luciferase were 8.65 mol/L and 11.9 mol/L, respectively, from the double reciprocal of Lineweaver-Burk plot shown in Fig. 3.

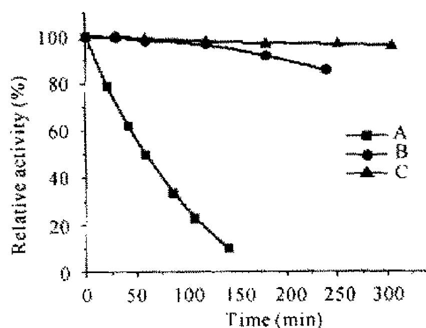

Fig. 2 Thermal stability of soluble enzyme and immobilized enzyme when they were incubated at 30 ℃ in 0.1 mol/L phosphate buffer pH 7.0

A: soluble enzyme; B: immobilized enzyme; C: immobilized enzyme, containing 4 mmol/L DTT

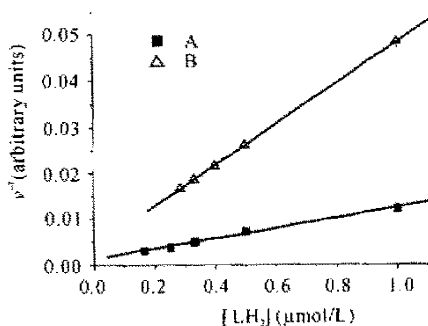

Fig. 3 Initial rate (v) of luciferase as a function of [LH_2] (μmol/L) in the Lineweaver-Burk coordinates.

A: soluble enzyme; B: immobilized enzyme

3) Optimum temperature and optimum pH for enzyme activity

According to procedure analogous to that for enzyme assay, the light intensities of Sepharose bound luciferase suspended in 0.02 mol/L Tris buffer, pH 7.8, containing 2 mmol/L EDTA were measured for variable temperature. The results are shown in Fig. 4. The optimum temperature was equal to 25℃, which was consistent with those for soluble and immobilized luciferase reported before. Similarly, the light peaks of immobilized and soluble enzyme were investigated at 25℃ under different pH conditions. The results are shown in Fig. 5. The optimum pH of immobilized enzyme was 7.8, the same as that of soluble enzyme. This suggested that there was no apparent gradient of protons between the bulk phase and the microenvironment of the bound enzyme.

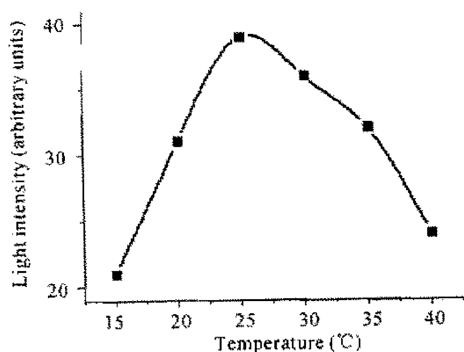

Fig. 4 Light intensity of immobilized enzyme as a function of temperature

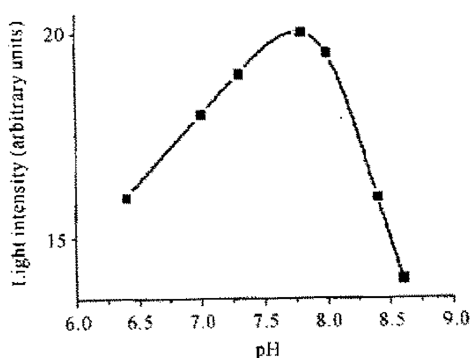

Fig. 5 Light intensity of immobilized enzyme as a function of pH

2. Fiber optic biosensor for ATP assay

1) Assay for ATP standard

Determination of ATP standards in different concentration was made by the procedure mentioned above. The peak in light intensity was reached within 1 min, longer than the response time obtained with soluble luciferase. Peak light intensity measured with the biosensor as a function of ATP concentration was plotted in log-log coordinates. The results are shown in Fig. 6. The biosensor responded linearly to ATP concentration from $10^{-8}-10^{-4}$mol/L.

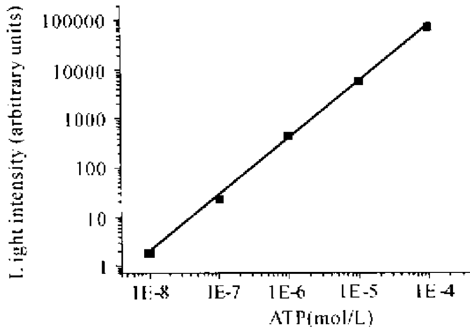

Fig.6 Calibration curve (log - log plot) for ATP assayed by the biosensor

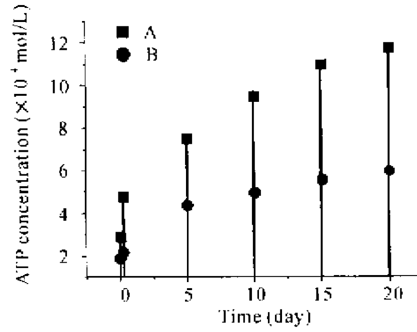

Fig.7 ATP concentrations change in serum from rats determined by the biosensor.
A: male rats; B: female rats

2) Assays for ATP in serum of rats bred in normal and low oxygen conditions

After preparation of rat blood samples as mentioned above, ATP concentrations in serum of rats were determined by the biosensor. We found obvious differences in serum ATP concentrations between rats bred in normal and low oxygen conditions. The normal serum ATP concentrations of female($n=6$) and male($n=6$) rats under normal oxygen condition were $1.89 \pm 0.35 \times 10^{-4}$ mol/L and $2.9 \pm 0.54 \times 10^{-4}$ mol/L respectively. The ATP content in serum of rats increased as time went on in low oxygen cabinet ($P < 0.05$). Fifteen days later, ATP concentration in female rats reached $5.58 \pm 1.07 \times 10^{-4}$ mol/L($n=6$), whereas that in male rats reached $4.8 \pm 0.91 \times 10^{-4}$mol/L($n=6$) after 6 hours and the concentration was up to $11.8 \pm 2.96 \times 10^{-4}$ mol/L ($n=6$) after 20 days. Under the same condition, the rate and level of increase in ATP content of male rats were higher than those of female ones ($P < 0.05$). It may be due to the difference of the regulation between two kinds of sex hormones.

The results showed that the presence of impurities, such as foreign proteins from crude luciferase extract, immobilized along with the enzyme, do not appreciably interfere with the ATP measurement. The biosensor, incorporating firefly luciferase luminescence system connected with luminometer by a fiber, was noted not only for being rapid and simple in the operation, but also for its sensitivity and good linear response for ATP assay.

References

Blum, L. J., Gautier, S. M., Coulet, P. R., 1988. Luminescence fiber optic biosensor. *Anal. Lett.*, **21**: 717 - 726.

Lagido, C., Pettitt, J., Porter, A. J. R., Paton, G. I., Glover, L. A., 2001. Development and application of bioluminescent *Caenorhabditis elegans* as multicellular eukaryotic biosensors. *FEBS Letters*, **493**: 36 - 39.

Leach, F. R., 1981. ATP determination with firefly luciferase. *J. Appl. Biochem.*, **3**: 473 - 478.

Lee, Y., Jablonski, I., Deluca, M., 1981. Immobilization of firefly luciferase on glass rods: Properties of the immobilized enzyme. *Anal. Biochem.*, **80**: 496 - 500.

Ji, X. S., Li, H. X., Yuan, Z. Y., Liu S. H., 1984. Immobilization of NAD kinase. Enzyme Engineering 7, *Anal. N. Y. Acad. Sci.*, **434**: 264 - 266.

Maria, P. X., Begona, V., Maria, D. M., 2000. Fiber optic monitoring of carbamate pesticides using porous glass with covalently bound chlorophenol red. *Biosensors & Bioelectronics*, **14**: 895 - 905.

Michel, P. E., Gautier-Sauvigne, S. M., Blum, L. J., 1998. A transient enzymatic inhibition as an efficient tool for the discriminating bioluminescent analysis of three adenylic nucleotides with a fiberoptic sensor based on a compartmentalized tri-enzymatic sensing layer. *Analytica Chimica Acta*, **360**: 89 - 99.

Neuberg, C., 1944. Convenient method for deproteinization. *Arch. Biochem.* **4**: 101 - 105.

Tservistas, M., Koneke, R., Comte, A. Scheper T., 2001. Oxygen monitoring in supercritical carbon dioxide using a fibre optic sensor. *Enzyme and Microbial Technology*, **28**: 637 - 634.

Vangelis, G. A., Yannis, D. C., 2002. A portable fiber-optic pesticide biosensor based on immobilized cholinesterase and sol-gel entrapped bromcresol purple for in-field use. *Biosensor & Bioelectronics*, **17**: 61 - 69.