Vol. 205, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1994 Pages 962-966

APPEARANCE OF HIGH ENZYME ACTIVITY IN IMMOBILIZED UREASE DISC BY ELECTRON BEAM IRRADIATION TECHNIQUE

M. KUMAKURA

Department of Bioscience, The Nishi-Tokyo University,
2525 Uenohara, Kitatsuru, Yamanashi 409-01, Japan

Received October 5, 1994

SUMMARY: A new preparation method of immobilized urease discs for biomedical applications, which was a thin circular film (200 μm,50mm ϕ), was developed. The method was achieved by electron beam irradiation of polyethyleneglycol diacrylate monomers in the addition of paper disc and bean powder as protective substance for irradiation by which a denaturation of the enzyme by irradiation was effectively prevented. The immobilized enzyme disc with a high enzyme activity (remaining activity yield), about 90%, was obtained. The enzyme activity was varied by the preparation conditions such as the thickness of paper disc, monomer concentration etc. The enzymes were trapped near the surface of the disc to be easily reacted with substrate. The trapped state of the enzymes appeared to be affected by a hydrophilicity of the polymers.

Urease is one of the most important enzymes in biomedical field. Therefore, its immobilization has been investigated by many workers on the relations of biomedical applications such as urease biosensor, artificial kidney etc.(1-8). However, to retain a high enzyme activity by immobilization was difficult because urease was very unstable and sensitive for chemical and physical actions. Enzymes have been successfully immobilized by chemical and physical methods(9,10). These methods have, however, had some inherent disadvantage or have presented difficulties such as partial deactivation of enzymes. One way to overcome such disadvantages is the entrapment of enzymes in polymer matrix. The immobilization of urease by entrapment which is known to be a mild way which has been tried, although enzyme reactions are resricted by diffusion of substrate(9). Allcock et al. have studied the entrapment immobilization of urease using γ-rays, in which the irradiation of urease solution containing monomer was impossible due to the denaturation of the enzymes by irradiation(8). The author has developed a new immobilization method of urease in the addition of soybean powder using low energy electron beam irradiation technique.

MATERIALS AND METHODS

Materials: Urease from jak bean (1500 units/g) was obtained from Wako Chemical Co., Ltd. Diethyleneglycol diacrylate (A-2G), Tetraethyleneglycol diacrylate

0006-291X/94 $5.00

(A-4G), Nonaethyleneglycol diacrylate (A-9G), and Tetradecaethyleneglycol diacrylate (A-14G) which were used as monomer were obtained from Shin-Nakamura Chemical Co., Ltd. Filter paper (whatoman No. 1) which was obtained from Tokyo Roshi Co., Ltd. was used as a paper disc. Microtest plate (96 holes, 6 mmϕ) which was obtained from Sumitomo Bakelite Co., Ltd. was used as a vessel in the irradiation. Bean powder (size, about 100 mesh) was prepared by the milling of soybean which was heat-treated at 200 ℃ for 40 min.

Preparation of immobilized urease discs: Paper discs (5 mmϕ) were obtained by cutting the filter paper using a punching machine. One piece of the paper discs was put into each hole of the microtest plate. The monomer solution containing urease (0.05%) and 0.04 M phosphate buffer (pH 7.2) containing 0.2% arabic gum and 0.6 M sodium tartarate coated on the paper disc in the hole by using a microsyringe. This microtest plate was irradiated in nitrogen gas atomoshere by low energy electron beam accelerator ("Curetron", Nissin-High Voltage Co., Ltd.), in which electron beam acceleration voltage and current were 300 keV and 5 mA, respectively. The irradiation was carried out with a belt-conveyer equipment of the accelerator and the speed of the belt-conveyer was fixed at 12 m/min. Various irradiation doses were performed by the repetition of the belt-conveyer in a certain stroke. After irradiation, the immobilized enzyme discs obtained by radiation polymerization take out from the microtest plate were washed several times with the phosphate buffer.

Measurement of enzyme activity: Enzyme activity of the immobilized enzyme disc and free enzyme was determined by measureing the amount of ammonia formed in the enzyme reaction. Enzyme reaction solution mixed mith a piece of the immobilized enzyme disc or the free enzyme solution and urea (0.2 %) in 0.04 M phosphate buffer containing 0.2 % arabic gum and 0.6 M sodium tartarate. Batch enzyme reaction was carried out at 30 ℃ for 30 min. After enzyme reaction, ammonia formed was determined with Nessler's methd. Enzyme activity (remained activity yield, %) of the immobilized enzyme disc was expressed as the ratio of ammonia formed in the immobilized enzyme disc to the free enzyme.

RESULTS AND DISCUSSION

Effect of the addition of the bean powder in the irradiation: Effect of the irradiation dose in the irradiation of urease solution with the bean powder was investigated as shown in Fig. 1. The enzyme activity of urease in the irradiation without the bean powder was decreased with increasing the irradiation dose, indicating that urease was sensitive for the irradiation. On the other hand, the decrease of the enzyme activity in the irradiation with the bean powder was limitted, when the irradiation dose was increased up to 10 Mrad. This result indicates that the bean powder is effectively played as a protective substance for the denaturation of the enzyme by radiation. Such a protective effect of the bean powder on the irradiation of urease was not known so far. The bean powder contains various substances such as globulin, albumin, enzymes(urease), amino acids. The bean powder used was heat-treated to eliminate the contribution of the enzymes contained. The protective effect of the bean powder was not varied by the concentration of the bean powder as seen in Fig. 1.

Effect of the component of the bean powder in the irradiation: The protective effect in the bean powder was examined by using the dissolved and undissolved component in water(Fig. 2). The enzyme activity in the addition of the dissolved component was not changed by the bean powder concentration, while that in the addition of the undissoled component was increased, indicating that

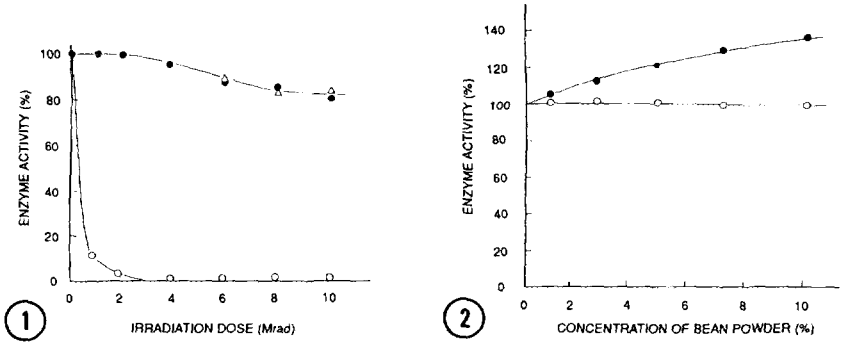


Figure 1. Effect of irradiation dose in the irradiation of urease solution(0.05%) with bean powder at 25℃. The concentration of bean powder: 0%(○), 1%(●), 5%(△).

Figure 2. Effect of the component of bean powder in the irradiation of urease solution(0.05%) at 25℃. The undissoluve component (●)and dissoluve component (○)were separated by centrifugation method (10,000rpm, 15min) of the bean powder solution.

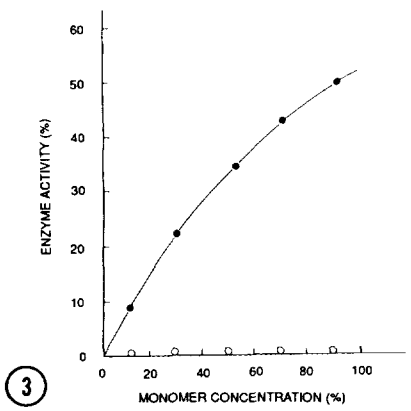
the dissolved substances contained in the bean such as proteins, globulin, amino acids are not contributed to the protection of urease in the irradiation.The undissolved substances composing the tissue of the bean are crosslinked atate with high molecular weights leading to network stracture. Urease should be adsorbed into such structures to be protected for chemical and physical actions. Thus, it is found from Figs. 1 and 2 that the protective effect of the bean powder in the irradiation is due to the adsorption of urease by the bean powder.

Effect of monomer concentration in the immobilization: As mentioned above, the author clarified that the enzyme activity of urease in the electron beam irradiation with 1 Mrad at room temperature can be remained by the addition of the bean powder. This made it possible to immobilize urease by the electron beam irradiation with the irradiation dose of 1 Mrad. The immobilized ureae discs with and without the bean powder were prepared by various monomer concentrations and their enzyme activities were examined as a function of monomer concentration as shown in Fig. 3. The enzyme activity of the immobilized urease disc with the bean powder was increased with increasing monomer (A-14G) concentration. On the other hand, the immobilized urease disc without the bean powder did not exhibit the enzyme activity due to presumably radiation denaturation. It is a significant result to get a high enzyme activity at high monomer concentrations such as 90%. So far, the enzyme activity of the enzymes immobilized by usual entrapment method was affected by the density of of the polymer matrix to be decreased due to the diffusion restriction of substrate and the decrease of the mobility of enzyme entrapped(9). This result means that the enzymes are effectively trapped on

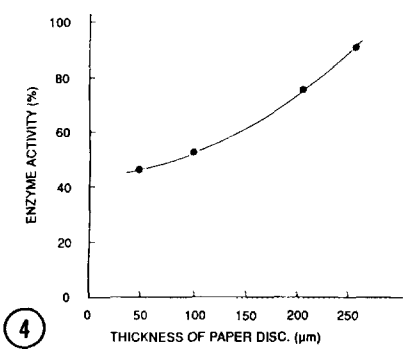
the surface of the fibrils of the paper disc to be delocarized, in which the polymer plays a role of adhesion reagent. The presence of the paper disc in the present method was, thus, important to reveal the enzyme activity.

Effect of the thickness of the paper disc: The enzyme activity of the immobilized urease disc increased with increasing the thickness of the paper disc as shown in Fig. 4. As increasing the thickness of the paper disc, the surface area of the immobilized urease disc is increased due to the decrease of the amount of the polymer matrix covering on the paper disc. Such an increase of the surface area leaded to the increase of the appearance of urease molecule from the polymer matrix. The enzyme activity of the immobilized urease disc with paper thickness of 250 μm gave about 90%, meaning that the state of urease immobilized is played like a free state. Since such a high remained activity yield in immobilized enzymes prepared by entrapment method was one of the characteristics of the present immobilization technique using electron beam accelerator. A high remained activity yield indicates that most of the enzymes used for the immobilization is trapped, not leaked, and also they are located on the surface of the fibrils covered with the polymer matrix to react efficiently with the substrate. This was able to achieve by low energy electron beam irradiation method only. The durability of the immobilized enzyme disc keeping high enzyme activity was confirmed by repeated batch enzyme reactions.

Relationship between enzyme activity and the nature of the monomers: Various polyoxyethyleneglycol diacrylate monomers were used for the immobilization, and the relationship between enzyme activity and number(n) of oxyethylene



③



④

Figure 3. Effect of monomer(A-14G) concentration in the immobilization of urease(0.025%). Irradiation: 1Mrad at 25℃. Bean powder concentration: 0%(○), 1%(●). Paper disc thickness: 100 μm.

Figure 4. Effect of the thickness of paper disc in the immobilization of urease(0.025%). Irradiation: 1Mrad at 25℃. Bean powder: 1%. Monomer: 90% A-14G.

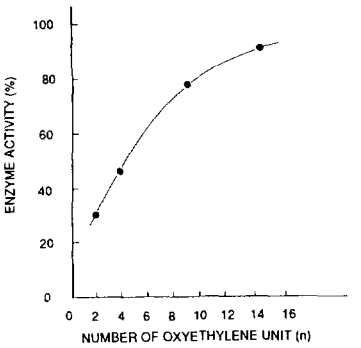


Figure 5. Relationship between enzyme activity and the number of oxyethylene unit of the monomers in the immobilization of urease(0.025%). Monomer concentration: 90%. Irradiation: 1Mrad at 25℃. Bean powder:1%. Paper disc: 200 μ m.

unit, $-CH_2CH_2O-$, was examined as shown in Fig. 5. The enzyme activity was increased with increasing the number of the oxyethylene unit, indicating that the appearance of the enzymes is increased with increasing the hydrophilicity of the polymer matrix resulting in the increase of the mobility of the enzyme molecule, in which the hydrophilicity of was due to the ether bond of the monmers. Such hydrophilic monomers were quickly polymerized by the electron beam irradiation with very short times (10—30 sec) to immobilize the enzymes. This high speed preparation of the immobilized enzyme discs was achieved by the selection of optimum polymerization condition and this was necessary to fix the enzymes in the suitable state for the enzyme reaction. The immobilized urease disc prepared was a small thin film form, though various sizes were able to prepare, and their porosity is large. Therefore, these discs could use for the biomedical applications such as urea assay kit, biosensor, artificial kidney etc. Furthermore, new this technique developed could apply for the immobilization of various enzymes.

REFERENCES

1. Butt, S.B. and Cammann, K. (1992) Anal. Lett. 9, 1597-1615.
2. Kallury, K.H.R., Lee, W.E., Thompson, M. (1992) Anal. Chem. 64, 1062-1068.
3. Thompson, J.C., Mazoh, J.A., Hochberg, A. Tseng, S.Y. and Seago, J.L. (1991) Anal Chem. 194, 295-301.
4. Kennedy, J.F., barker, S.A. and Pike, V.W. (1977) Biochem. Biophys. Acta 484, 115-126.
5. Bowers, L.D., Canning, L.M.Jr., Sayers, C.N. and Carr, P.W. (1976) Clin. Chem. 22, 1314-1318.
6. Kitano, M., Yoshijima, S., Motogi, S. and Ise, N. (1980) Biotechnol. Bioeng. 22, 2633-2642.
7. Kitano, M., Yoshijima, S. and Ise, N. (1980) Biotechnol. Bioeng. 22, 2643-2653.
8. Allock, H.R., Pucher, S.R. and Visscher, K.B. (1994) Biomaterials 15, 502-506.
9. Woodward, J., Ed., (1985) Immobilized Cells and Enzymes, A Practical Approach, IRL Press, Oxford.
10. Hartmeir, J., Ed., (1988) Immobilized Biocatalysts, Springer-Verlag Berlin Heidelberg.