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Preparation of polymeric urease discs by an electron beam irradiation technique

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A preparation method of immobilized urease discs by an electron beam irradiation technique was developed, and the relationship between enzyme activity and preparation conditions was investigated. The immobilized urease disc was a thin circular film (200 μm, 5 mm ϕ) that is useful for biomedical applications. The activity of urease irradiated with 1 Mrad at room temperature was protected by the presence of cysteine. The activity of the immobilized urease discs was studied as a function of monomer concentration (80–90%) and the thicker disc gave a high activity. The durability of the immobilized urease discs gave a high activity. The durability of the immobilized urease discs was evaluated by repeated batch enzyme reactions, and a high activity yield (80–85%) was obtained.

Keywords: Urease, immobilization, electron beam irradiation, protective agent

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Urease is one of the most important enzymes in biomedical applications. Hence, its immobilization has been investigated by many workers in relation to the urease biosensor and artificial kidneys[1–8]. Enzymes have been successfully immobilized by chemical or physical methods[9,10]. However, these methods have some inherent disadvantages or have presented difficulties such as partial deactivation of the enzymes. One way to overcome such disadvantages is the entrapment of the enzymes in polymers.

A potentiometric urea biosensor was based on a modified poly(vinyl chloride) (PVC)–nonactin ammonia-sensitive gas electrode, in which urease was physically entrapped between a special thin gas-permeable membrane and a dialysis membrane[11]. Longer response times were observed in the case of the PVC–nonactin membrane electrode when it was not sufficiently pressed over the gas membrane. Hollow-fibre enzyme reactors, in which urease was entrapped in hollow fibres, have been studied for the artificial kidney[6,7,9]. Immobilization within a hollow fibre can be achieved by leading the enzyme solution into the hollow core by capillary action and then sealing the two ends. The half-life of the entrapped urease in the continuous reaction system is usually lower than that of the free enzyme. The diffusion of the substances in the immobilized enzyme obtained by the entrapment method is important and thus the enzyme gel beads immobilized by the entrapment method have been studied[9,10].

We have studied the immobilization of microbial cells and antibodies by radiation polymerization with γ-rays[12,13]. So far, the entrapment of urease using γ-rays has been studied by Allcock *et al.*[8]. However, to get the immobilized urease, the irradiation of urease solution containing the monomer was not carried out because urease was very sensitive to irradiation in the solution state[8]. In this work, the preparation of immobilized urease discs with large surface areas was developed by using an electron beam accelerator, in which urease solution containing the monomer was irradiated in the presence of a protective reagent.

MATERIALS AND METHODS

Materials

Urease from jack beans (1500 units g^{-1}) was obtained from Wako Chemical Co., Japan. Tetradecaethyleneglycol diacrylate (A-14G), which was used as monomer, was obtained from Shin-Nakamura Chemical Co., Japan Filter paper (Whatman No. 1) from Toyo Roshi Co., Japan, was used as a porous base material. A microtest plate (96 holes, 6 mm ϕ), obtained from Sumitomo Bakelite Co., Japan, was used as a vessel in the irradiation. Urea, cysteine and Nessler's reagent were obtained from Wako Chemical Co., Japan.

Immobilization of urease

Paper discs (5 mm ϕ) were obtained by cutting the filter paper using a punch machine. One paper disc was put into each hole of the microtest plates. The monomer (A-14G) solution containing urease (0.05%) and 0.04 M phosphate buffer (pH 7.2) containing 0.2% arabic gum

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and 0.6 M sodium tartrate were coated on the paper disc in the hole by using a microsyringe. This microtest plate was irradiated in a nitrogen gas atmosphere by a low-energy electron beam accelerator ('Curetron'. Nissan-High Voltage Co., Japan), in which the electron beam acceleration voltage and current were 300 keV and 5 mA, respectively. The irradiation was carried out with the belt-conveyer equipment of the accelerator and the speed of the belt-conveyer was fixed at 12 m min^{-1}. Therefore, 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 taken out from the microtest plate were washed several times with the phosphate buffer.

Measurement of enzyme activity

The enzyme activity of the immobilized enzyme disc and the free enzyme was determined by measuring the amount of ammonia formed in the enzyme reaction. The enzyme reaction solution was mixed with a piece of the immobilized enzyme disc or the free enzyme solution and urea (0.2%) in 0.04 M phosphate buffer including 0.2% arabic gum and 0.6 M sodium tartrate. The batch enzyme reaction was carried out at 30°C for 30 min. After the enzyme reaction, the ammonia formed was determined by Nessler's method (reagent, 1 ml; developing time, 15 min; absorbance, 420 nm). The activity yield of the immobilized enzyme disc was expressed as the ratio of ammonia formed in the immobilized enzyme disc to that in the free enzymes.

RESULTS AND DISCUSSION

Effect of cysteine concentration

Before the immobilization of urease by irradiation, the optimum condition for the irradiation of urease was studied. It is known that urease is stabilized by SH compounds such as cysteine and the effect of cysteine concentration on the free enzymes at low temperatures was studied. The relationship between relative activity and cysteine concentration is shown in *Figure 1*. The relative activity at room temperature (25°C) increased and then slightly decreased on increasing the concentration of cysteine. On the other hand, the relative activity at low temperature (−78°C) increased, reached a maximum (cysteine, 5×10^{-3} M) and then rapidly decreased on increasing the concentration of cysteine. A protective effect by cysteine at room temperature was expected, but that at low temperature was a new observation. The decrease in the relative activity at the high concentration at −78°C indicated that cysteine reacted with urease to be deactivated. This deactivation reaction should result in the formation of a cross-linked complex of urease with cysteine, and the complex would be precipitated at low temperatures. From this result, it was found that urease was effectively protected by cysteine on irradiation, with an irradiation dose of 1 Mrad at room temperature.

Effect of irradiation dose

The effect of irradiation dose on enzyme activity was studied; urease solution was irradiated with various

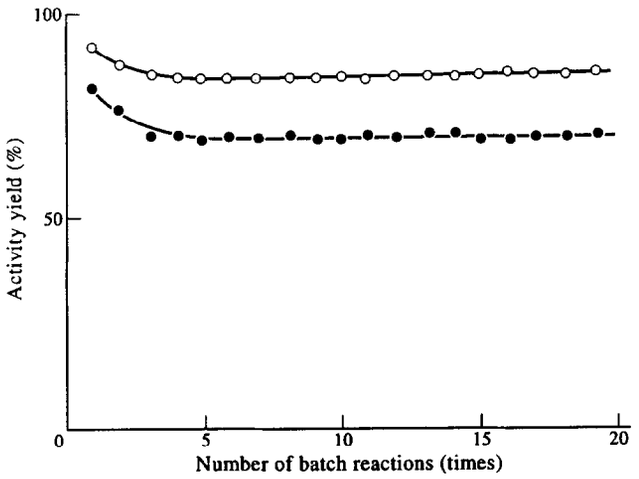

Figure 1 Effect of cysteine concentration in the irradiation of 0.05% urease. Irradiation dose: 1 Mrad. Irradiation temperature: ●, 25°C; ○, −78°C.

doses at various temperatures with and without cysteine. The irradiation without cysteine deactivated urease at low temperatures (−15, −78°C) even though enzymes are generally protected in the frozen state. In previous work on the irradiation effect of cellulase and α-glucosidase, the enzyme activity was not deactivated by irradiation in the frozen state (−24, −78°C), indicating that the enzymes were not deactivated by irradiation at lower temperatures[14,15]. The enzyme solution was frozen at low temperatures (below −10°C), at which the mobility of the enzyme molecule was fixed and isolated. At such low temperatures, the enzymes are precipitated from the system and this is similar to the solid state, in which the irradiation effect is not observed or is very small. The result in *Figure 2* shows that urease is very sensitive to irradiation and is deactivated even in the solid state. As can be seen in *Figure 2*, the activity of urease in the liquid state was enhanced with cysteine, and after that the activity was constant on increasing the irradiation dose at room temperature, meaning that urease was not deactivated by irradiation in the presence of cysteine (5×10^{-3} M)

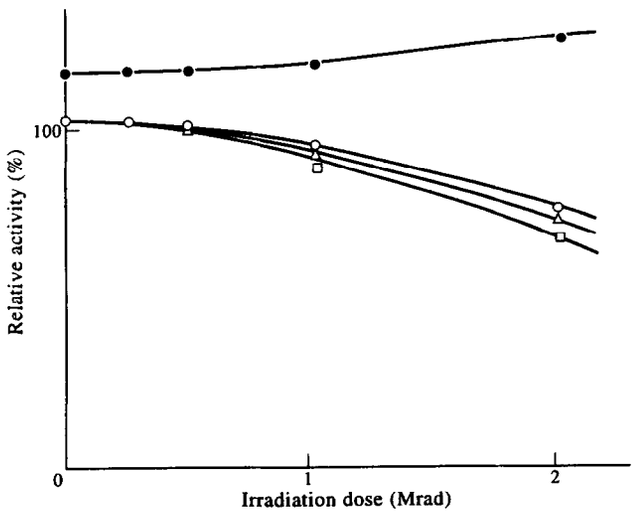

Figure 2 Effect of irradiation dose in the irradiation of 0.005% urease. With cysteine (5×10^{-3} M): ●, 25°C. Without cysteine: □, 25°C; △, −24°C; ○, −78°C.

within the range of irradiation doses examined. This suggested that irradiation with an electron beam can cause immobilization of urease. The electron beam accelerator was given a high irradiation dose rate, indicating that the irradiation is able to take place in a short time, about 10–30 s. This was useful for the immobilization of urease, which is sensitive to irradiation.

Effect of monomer concentration

The immobilization of urease by radiation was carried out in the presence of cysteine, based on the above results. The effect of monomer (A-14G) concentration on the activity yield of the immobilized enzyme disc was studied, and the relationship between activity yield and monomer concentration is shown in *Figure 3*. The activity yield of the immobilized enzyme disc with cysteine increased with increasing monomer concentration, but that of the immobilized enzyme disc without cysteine did not. This result was a significant observation, because the activity of urease irradiated even without cysteine was observed, as shown in *Figure 2*. This indicates that urease is also sensitive to organic compounds such as A-14G, which is a relatively non-toxic monomer with long oxyethylene units. In our previous work, we have used this monomer to immobilize trypsin by γ-irradiation at −78°C, but trypsin was not deactivated without a protective reagent[16]. From this result, it was found that the activity of urease was influenced by the property of the monomer in the absence of cysteine.

In *Figure 3*, it is interesting to note that the activity yield of the immobilized enzyme disc increased with increasing monomer concentration. In the immobilization of trypsin using A-14G, to get a high activity yield the optimum concentration of the monomer was about 50–60%, even though the irradiation was carried out at low temperature (−78°C)[16]. Therefore, the activity yield of the immobilized trypsin obtained with high monomer concentrations (above 80%) was low because trypsin was entrapped in the polymer matrix, resulting in the loss of enzyme mobility and the

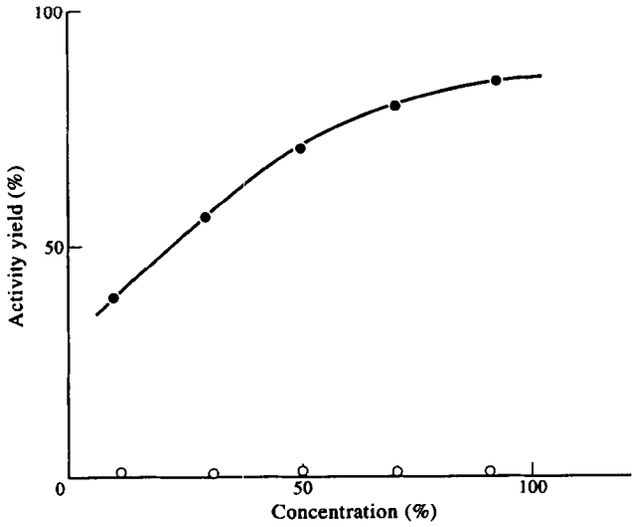

Figure 3 Relationship between monomer concentration and activity yield in the immobilized enzyme discs. Urease: 0.05%. Cysteine: 5×10^{-3} M. Monomer coating volume: 2 μl. Irradiation: 1 Mrad at 25°C.

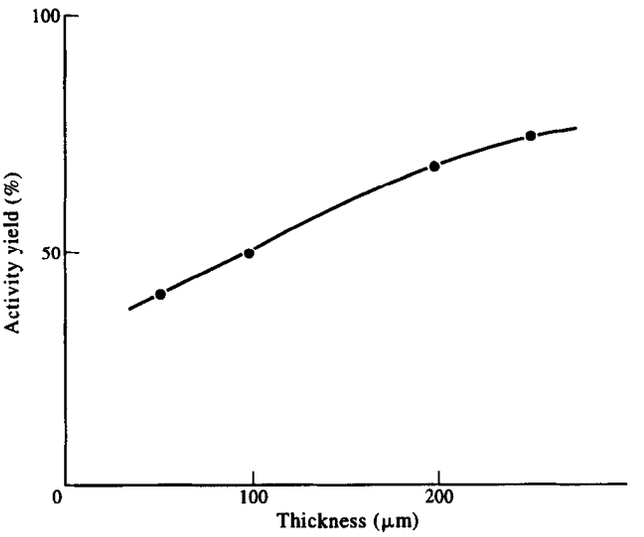

Figure 4 Relationship between the thickness of the paper disc and activity yield in the immobilized enzyme discs. Urease: 0.05%. Cysteine: 5×10^{-3} M. Monomer concentration: 50%. Irradiation: 1 Mrad at 25°C.

covering of the active site of the enzyme. The decrease of the activity yield at low monomer concentrations would be due to the leakage of the enzymes from the polymer matrix. The activity yield at high monomer concentrations in *Figure 3* means that the enzymes are effectively trapped on the surface of the fibrils of the paper disc to be delocalized, in which the polymer plays the role of an adhesion reagent. It was found that the presence of the paper disc in the present method was important to develop the enzyme activity in the immobilization method by irradiation at room temperature.

Effect of thickness of the paper disc

The effect of the thickness of the paper disc on the activity yield of the immobilized enzyme disc was studied. The activity yield of the immobilized enzyme discs, which were obtained with 50% monomer concentration, increased on increasing the thickness of the paper disc, as shown in *Figure 4*. On increasing the thickness of the paper disc, the thickness of the polymer matrix covering the surface of the paper disc is decreased, and this then leads to an increase in the amount of enzymes which appeared from the polymer matrix owing to the increase in the surface area. That is, the porosity of the immobilized enzyme disc increases on increasing the thickness of the paper disc, by which the diffusion resistance of the substrate into the polymer matrix is reduced. A-14G used in this work, which is a bifunctional monomer, was easily polymerized by electron beam irradiation with an irradiation dose of about 0.5–1 Mrad and gave a hydrophilic polymer gel by swelling in water. This polymer is a cross-linked structure because of the bifunctional monomer with two vinyl groups. The enzymes immobilized on the surface of the fibrils of the paper by such a polymer would adopt a tightly entrapped state.

Effect of coating volume of the monomer

The relationship between activity yield and coating volume of the monomer in the immobilization is

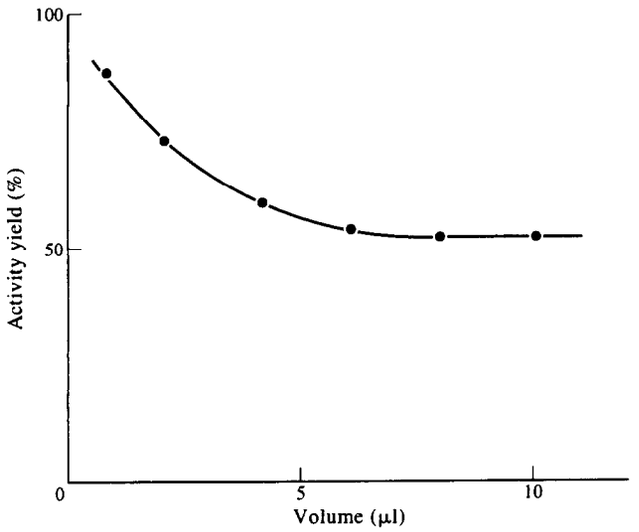

Figure 5 Relationship between the coating volume of the monomer and activity yield in the immobilized enzyme discs. Urease: 0.05%. Cysteine: 5×10^{-3} M. Monomer concentration: 50%. Irradiation: 1 Mrad at 25°C.

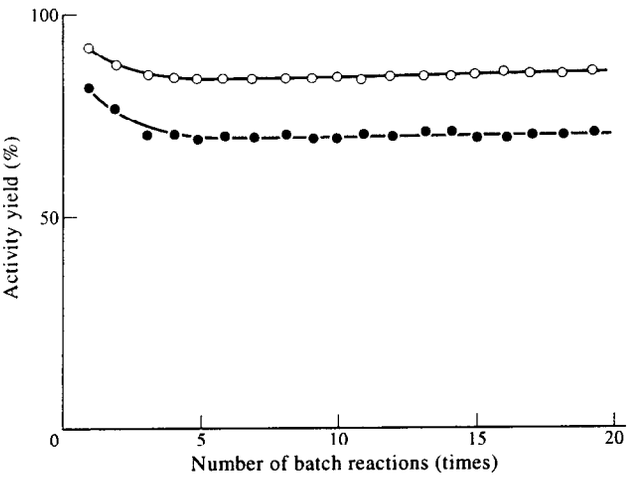

Figure 6 Repeated batch enzyme reactions in the immobilized enzyme discs. Urease: 0.5%. Cysteine: 5×10^{-3} M. Coating volume of monomer: 2 μl. Irradiation: 1 Mrad at 25°C. Monomer concentration: ●, 50%; ○ 90%.

shown in *Figure 5*. The activity yield of the immobilized enzyme disc decreased on increasing the coating volume, but the decrease of the activity yield was moderate at a high coating volume. The increase in the activity yield for a low coating volume corresponded to the decrease in the thickness of the polymer matrix on the paper disc. The coating volume of 2 μl on the standard size of the paper disc of thickness 200 μm and diameter 5 mm appeared to be optimum. To increase the coating volume, the paper disc was immersed and its surface was completely covered by the polymer matrix immobilizing the enzymes. It was interesting that the enzymes immobilized in such a bulky polymer matrix reveal this activity yield, suggesting that the enzymes are conveniently trapped on or near the surface of the polymer matrix, because radiation polymerization is performed at room temperature, resulting in the migration of the enzymes onto the surface from the inside of the polymer matrix, during the process of coating the monomer and the irradiation.

Durability of immobilized enzyme discs

The durability of the immobilized enzyme discs was examined by repeated batch enzyme reactions. The activity yields of the immobilized enzyme discs were constant throughout repeated batch enzyme reactions, as shown in *Figure 6*. The activity yield of the immobilized enzyme disc in a 50% monomer concentration was slightly lower than that in a 90% monomer concentration, but its value was kept for a long time, indicating that the enzymes are not leaked by repeated batch enzyme reactions. The advantage of the immobilization method of enzymes using the electron beam irradiation technique was a simple process and short time, in which the time necessary for the radiation polymerization (immobilization) was approximately 10–30 s depending on the conditions of irradiation. Many immobilized enzyme discs can be prepared by one process using many microtest plates with 96 holes per plate.

The standard size of the immobilized enzyme disc prepared by the electron beam irradiation technique is a thin circular film (200 μm × 5 mm ϕ). The immobilized enzyme discs prepared by the present method can apply to various fields; that is, one sample of the immobilized enzyme discs can be used for the assay of urea by biosensor. The column packed with the immobilized enzyme discs can also be used for the artificial kidney as an enzyme reactor in which the ion-exchange column to remove ammonium ions is connected.

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