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A NEW METHOD OF IMMOBILIZING ENZYMES BY RADIOPOLYMERIZATION UNDER LOW TEMPERATURE

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A new method of immobilizing enzymes by ionizing radiation is described. The mixed aqueous solution of enzyme and polymerizing reagents were quickly frozen at about −70°C then were irradiated with 200 to 500 Krad by ^{60}Co γ ray. Irradiation was conducted aerobically under the low temperature. The enzyme was entraped in the resulting polymer. As the polymerizing reagent some water soluble polymers having vinyl bonds were also applicable. By this method an immobilized enzyme was prepared in bead, membrane, bag, or tube form having high enzymic activity. When the bead form was to be prepared, the mixture of enzyme and reagents were injected into precooled solvent such as *n*-hexane, toluene, or petroleum ether. the size of the bead was controlled freely from 10 μm to 1 cm in diameter. The surface of the bead had numerous small holes and the cross section of the bead showed a spongy structure. some acrylates were suitable for the immobilization of enzymes which required the corresponding metal ion as the essential substance. Microorganisms and multienzymes will be immobilized by this technique. This method is inexpensive, quick, simple, and reliable. Immobilized microbial cells can be sterilized by γ irradiation. Invertase was immobilized and the application test was conducted in an enzyme column.

INTRODUCTION

There are many methods of immobilizing enzymes. These methods are usually divided into five groups: Covalent binding, entrapment, cross-linking, adsorption, and microencapsulation.

Entrapment is one of the most popular methods. The work reported by Bernferd and Wan (1) was the first of many such publications. They used acrylamide as their polymerizing reagent.

In the entrapment method, a substance is polymerized and the enzyme is entraped in the resulting polymer. Thus any substance is applicable provided it is water soluble, can be polymerized, and is not harmful to enzymes. The compounds acrylamide (2–4) *N,N'*-methylenebisacrylamide (5), 2-hydroxyethyl methacrylate (6), collagen (7), starch (8), silicon rubber

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(9,10), silics gel (11), and agar (12) are such materials. Among these materials acrylamide is favored most often because of its ease of handling and reliability. It is well known that some chemical reagents are polymerizable by ionizing radiation such as γ radiation, X radiation and electron acceleration. Dobo (13) reported a method in 1970 of polymerizing acrylamide by X irradiation. He mixed tripsin, acrylamide, and *N,N'*-methylene bisacrylamide in a glass ampul and deaerated by bubbling purified argon. After irradiating with 3 Krad he found a precipitate containing enzyme.

The merits of polymerizing reagents by ionizing radiation can be summarized as follows:

(1) Polymerization can be accomplished at any desired temperature, especially at low temperatures.
(2) Solid as well as liquid substances can be activated. Solid phase substances at low temperatures will be polymerized only by ionizing radiation.
(3) Wide varieties of chemical substances are applicable as polymerizing reagents. Besides acrylamide and *N,N'*-methlenebisacrylamide, acrylic acid, acrylates, methacrylic acid, methacrylates, vinylpyrrolidone, polyvinylpyrrolidone, polyvinyl alcohol, polyethylene glycol dimethacrylate, divinyl sulfone, and so on will be successful.
(4) The resulting polymers do not contain chemical catalysts and enzymes will not be inactivated by them.
(5) Entraped microorganisms will be sterilized retaining enzymic activities.
(6) The initiation and completion of the polymerization process can be precisely controlled.

RADIOPOLYMERIZATION AT LOW TEMPERATURES

In Japan, enzyme entrapment by ionizing radiation was first attempted 1973 by Kawashima and Umeda (14) and Maeda, Suzuki, and Yamauchi (15). Yoshida, Kumakura, and Kaetsu also reported similar work (16).

Kawashima has developed a new method in which the enzyme is mixed with polymerizing reagents, quickly frozen, and then γ-irradiated under aerobic conditions. There are four purposes for radiopolymerization at low temperatures.

First, by the quick freezing of the enzyme–polymerizing reagents mixture, water will separate as small ice crystals and the polymerizing reagents condense around the ice crystals. After γ-irradiation the enzyme is entraped in the resulting polymer. When it is melted the ice crystals disappear and a spongy texture results. The small pores which make up the spongy texture are about 2 to 10 μm in diameter.

Immobilized enzymes prepared by the entrapment method commonly have a low surface area. To obtain large surface areas immobilized enzymes are cut into small pieces. Sometimes the enzyme is entrapped along with a carrier such as cotton wool or some other textile. But those prepared by radiopolymerization at low temperatures have a large surface area without any additional carriers. The spongy texture changes according to the freezing temperature and polymerizing reagents employed (Figs. 1–3).

Second, by the use of the freezing process, various forms of immobilized enzymes are obtainable. Beads, membranes, or even bag-shaped immobilized enzyme derivatives can be prepared corresponding to the frozen shape before radiopolymerization.

Third, at low temperatures the inactivation of enzymes due to polymerization heat will be reduced.

Fourth, radiation inactivation of enzymes is not as serious at low temperatures.

Maeda, Suzuki, and Yamauchi polymerized polyvinyl alcohol (17) and polyvinyl pyrrolidone (18) as well as acrylamide (15) under anaerobic conditions at ambient temperatures with high doses.

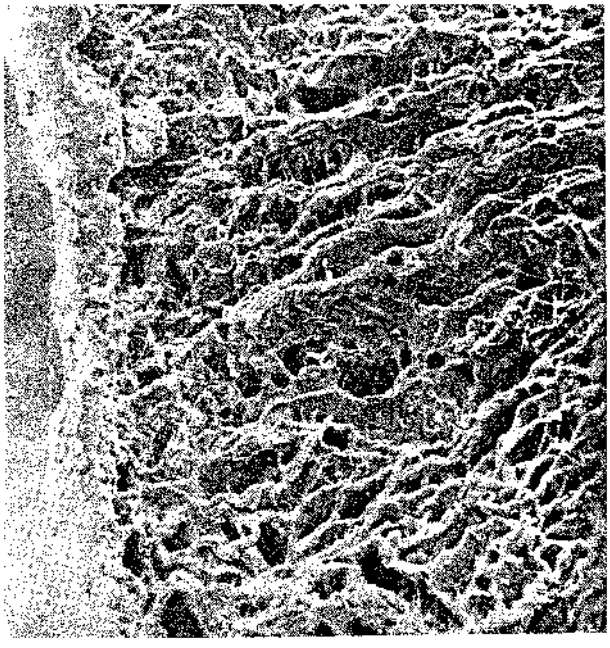

FIG. 1. Spongy structure of immobilized enzyme preparation (20) (×300). Polymerizing reagents: AA:enzyme is 2:1 by volume (AA = 30% acrylamide containing 2.7% *N,N'*-methylenebisacrylamide).

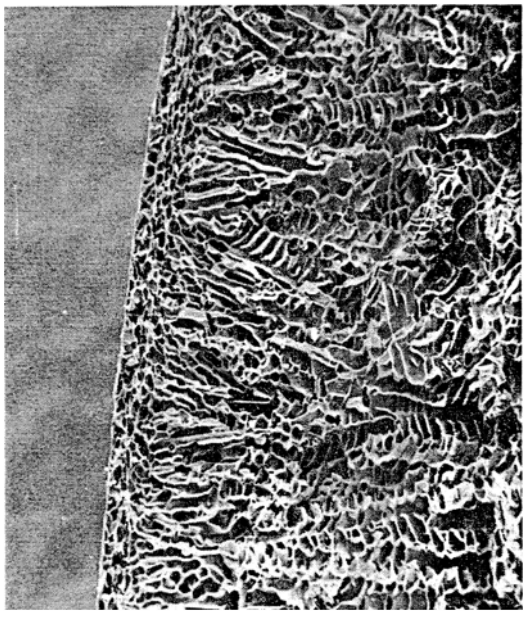

FIG. 2. Spongy structure of immobilized enzyme preparation (20) (×300). Polymerizing reagents: AA : ANa : ACa : enzyme is 1 : 1 : 1 : 1 (AA is described in Fig. 1. ANa = 30% sodium acrylate and ACa = 30% calcium acrylate).

PREPARATION OF THE IMMOBILIZED ENZYMES

Membranous Immobilized enzymes (19–21)

A mixture of enzyme and polymerizing reagents (5 ml) are put into a flask which is immersed in a coolant such as dry ice acetone and quickly rotated. In most cases the following mixture is favored: 30% sodium acrylate : 30% calcium acrylate containing 2.7% *N,N'*-methylene-bisacrylamide : enzyme in a ratio of 1 : 2 : 1 : 1 by volume. On the inner wall of the flask a thin frozen film of enzyme–reagents mixture is formed. After γ-irradiation (50 to 100 Krad, preferably 200 to 500 Krad), the frozen film is melted at room temperature and a membranous immobilized enzyme obtained (Fig. 4). There will be little difference in the enzyme activity whether the frozen film is defrosted immediately after irradiation or defrosted after being kept in the frozen state overnight. The film is then cut to proper size and washed with water or buffer. As the cross-linking reagent, divinylsulfone and polyethyleneglycol dimethacrylate can be used besides *N,N'*-methylenebisacrylamide. In this case the final concentration should be 0.5 to 2%.

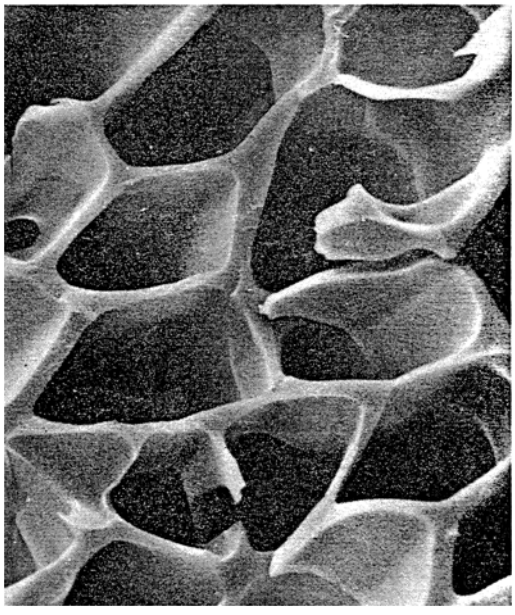

FIG. 3. Spongy structure of immobilized enzyme preparation (20) (×3000). Polymerizing reagents: AA : ACa : enzyme is 2 : 2 : 1 (AA and ACa are described in Figs. 1 and 2).

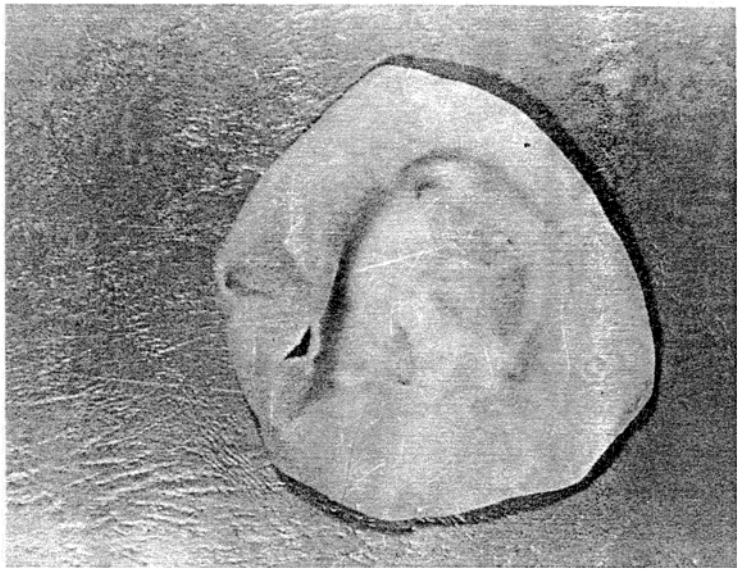

FIG. 4. Membranous immobilized enzyme.

Bead-Shaped Immobilized Enzymes

Bead-shaped immobilized enzymes are favored for use in enzyme columns. Nilsson, Mosbach, and Mosbach (22) reported bead polymerization of acrylamide in toluene-chloroform. However the resulting polymer is rather soft and the bead size is too small (10 to 250 μm).

In our method, bead-shaped immobilized enzymes can be prepared easily (23–25). The mixed solution of enzyme and polymerizing reagents are injected into precooled solvent and then γ-irradiated. The resulting bead polymer has a spongy structure (Fig. 5). It is not necessary to minimize the bead size to increase the surface area. The size of bead can be controlled from about 10 μm to 1 cm (Fig. 6). The polymer proved to be as physically stable as commercial porous resins depending on the polymerizing reagents employed.

As the freezing fluid an organic solvent such as *n*-hexane or toluene are favored (Table 1). After irradiating with an adequate dose, the solvent is immediately discarded and the frozen polymer melted in ice water or proper buffer.

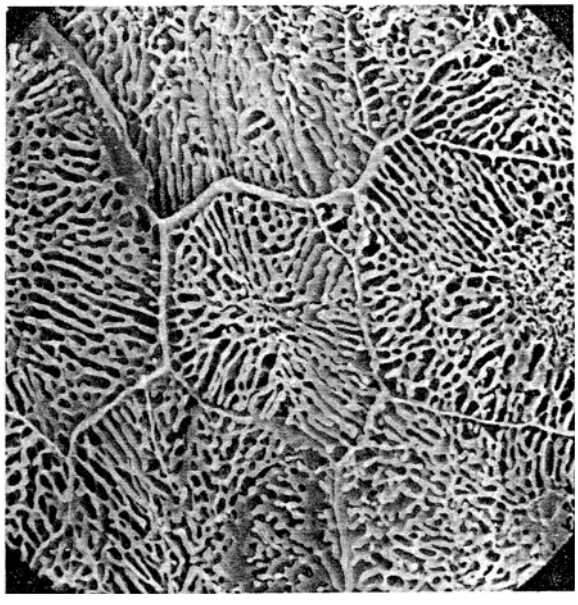

FIG. 5. Scanning electron microscopic view of bead surface (23) (×1000).

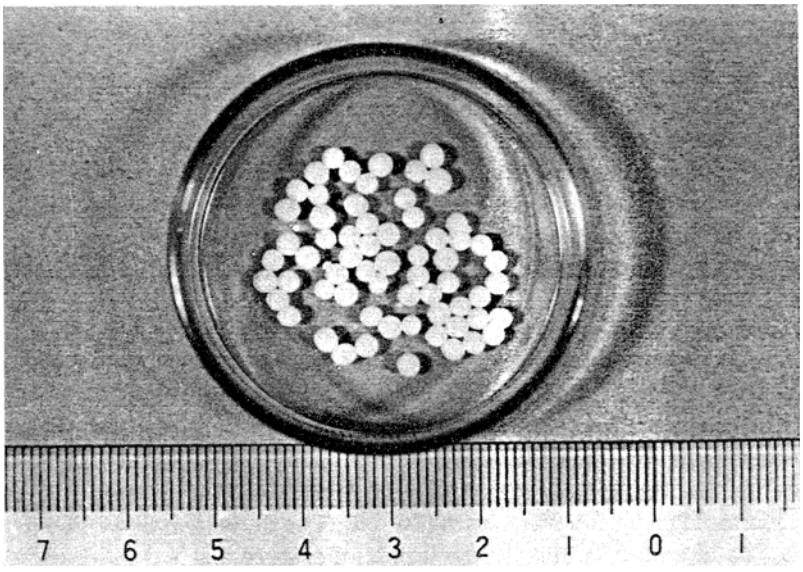

FIG. 6. Bead-shaped immobilized invertase (23).

TABLE 1. **Effect of Freezing Fluid on the Bead Formation and Activity of Immobilized Enzyme (23)**

Fluid	Bead formation	Activity[a]
n-hexane	Bead formed	100
Petroleum ether	Bead formed	100
Toluene	Bead formed	100
Xylene	Bead formed	98
Ethyl ether	Bead formed	94
Methylethylketone	Bead formed	86
Butanol	Bead formed	80
Ethyl acetate	Bead formed	80
Soybean oil	Bead formed	75
Amyl alcohol	Massive gel formed	95
Benzene	Massive gel formed	95
Trichloroethylene	Massive gel formed	90
Carbon tetrachloride	Massive gel formed	95
Methanol	No polymer formed	0
Ethanol	No polymer formed	20
Propanol	No polymer formed	0
Acetone		0

[a] Activity of invertase immobilized in *n*-hexane was assumed to be 100.

IMMOBILIZATION OF INVERTASE

The physical properties of acrylate polymers vary with the salt used. The polymer of sodium or potassium acrylate has higher enzymic activity but it is too soft since it absorbs much water. The entraped enzyme is apt to leak from the polymer. In contrast the polymer of calcium or magnesium acrylate has low enzymic activity but is physically stable. We recommend mixing several polymerizing reagents according to the enzyme.

Some enzymes require Ca^{2+}, Mg^{2+}, Na^{+} or K^{+} as the essential element. In this case the corresponding acrylate is desirable in order to prepare an immobilized enzyme of high activity. We found that the retained activity of immobilized invertase varied, depending on the reagent combinations employed (Table 2). The acrylate polymer is cationic so that the optimum pH of the immobilized invertase was shifted toward the acidic side by 0.7 to 1.0 units (invertase was immobilized according to No. 5 in Table 2). The optimum reaction temperature and heat stability of the invertase decreased after immobilization in most cases (26–28). The derivative described in this report has a greater optimum reaction temperature and was more stable to the heat treatment in the buffer than in the soluble enzyme. The retained activity was about 20 to 91.5% according to enzyme concentration (Fig. 7) and on bead size (Fig. 8). The smaller bead has a large surface area and this reflects its higher enzymatic activity. The leakage of the immobilized enzyme was about 2 to 5% and the value was fairly constant regardless of the enzyme concentration in the polymer. Several proteins were entrapped and examined for enzyme leakage (21). As Figure 9 shows, the protein of lower molecular weight was apt to leak more. In order to check the leakage stability, membranous immobilized invertase was used up to 30 times,

TABLE 2. **Retained Activities and Swelling Ratio of Invertase Immobilized at Various Monomer Combinations (21)**

No.	AA (ml)	ANa	ACa	Enzyme in solution[a]	Activity (%)[b]	Swelling ratio[c]
1	2	2	0	1	45.2	2.1
2	3	0	1	1	39.5	1.7
3	2	1	1	1	45.5	2.5
4	1	2	1	1	49.3	3.9
5	1	1	2	1	48.2	2.6
6	2	0	2	1	41.1	1.1

[a] Invertase 2 mg/ml.
[b] Enzyme activity after immobilization was compared with the activity before immobilization.
[c] Immobilized enzyme preparation was weighed before (A) and after (B) absorbing water overnight at room temperature and B/A was calculated.

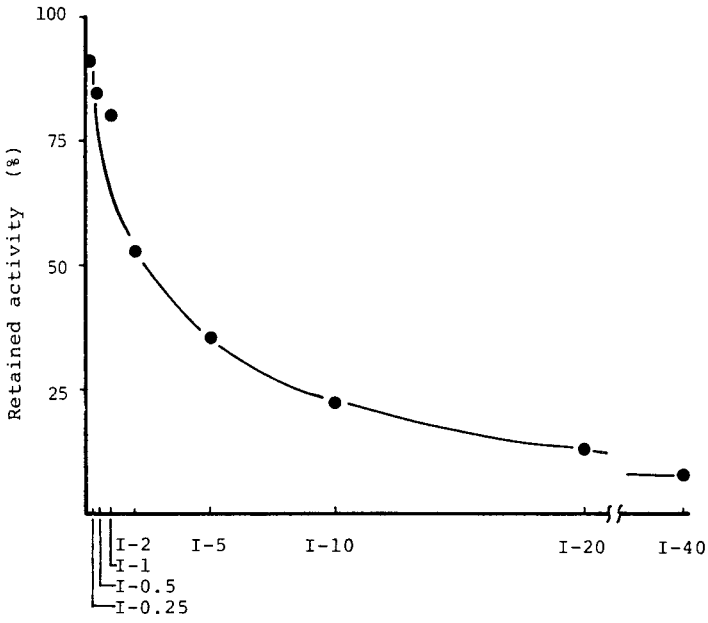

FIG. 7. Relation between the retained activities and enzyme concentrations of immobilized invertase (29). Each preparation was cut finely and a part of it (1/500 to 1/50) subjected to activity assay. *I-n* means an immobilized enzyme prepared from 5 ml of enzyme-polymerizing reagents containing *n* mg of invertase.

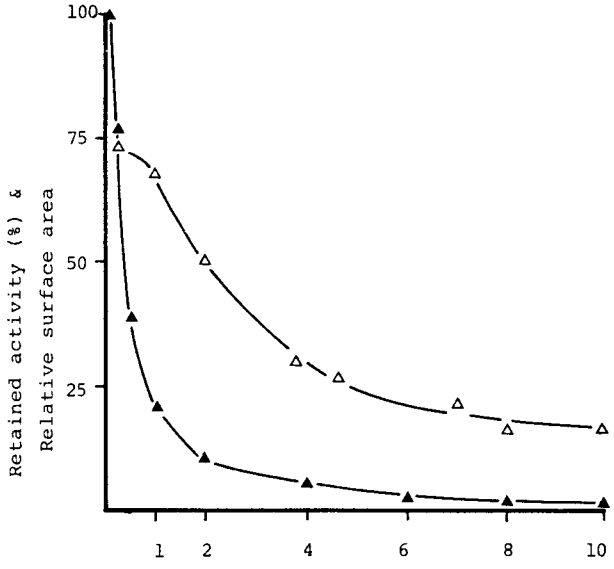

FIG. 8. The effect of bead size on the retained activities of bead-shaped immobilized invertase (23). —△—, retained activity; —▲—, relative surface area. Beads were prepared from an enzyme monomer solution containing 0.4 mg of invertase/ml.

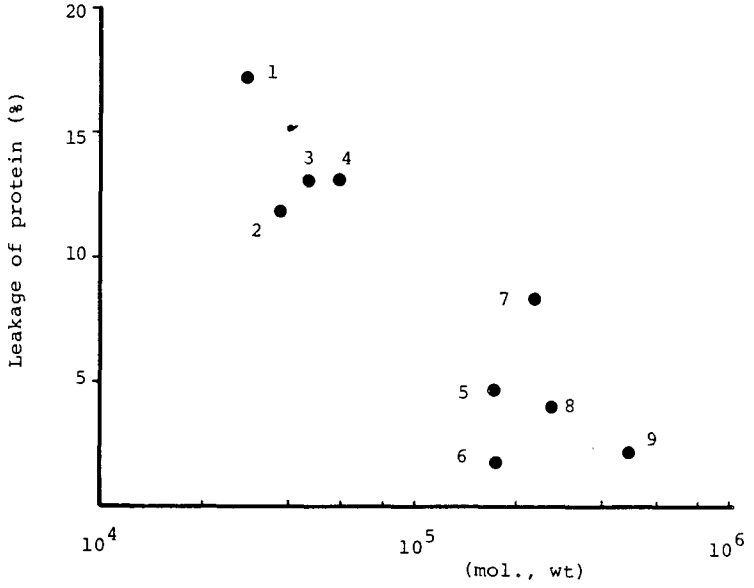

FIG. 9. The effect of molecular weight of immobilized protein on the leakage (21). Of each protein 25 mg was mixed with 5 ml of monomer solution (AA : ANa : ACa : water = 1 : 1 : 2 : 1) and immobilized. Each preparation was shaken with 100 ml of water overnight at 30°C and the amount of protein released was determined by an amino acid autoanalyzer. AA, ANa, and ACa are described in Figs. 1 and 2.

repeatedly (29). Activity loss was about 1% with every usage (Fig. 10). The preparation was quite stable in a dry state and retained 60 to 90% of its original activity even after storage at 35°C for 90 days. There were no large differences between membranous and beaded immobilized invertase. The K_m value for the native enzyme was 1.70×10^{-2} M and 1.95×10^{-2} M for the membranous immobilized invertase or 2.45×10^{-2} M for the bead-shaped immobilized invertase.

APPLICATION TEST OF IMMOBILIZED INVERTASE

Immobilized invertase prepared according to No. 5 of Table 3 was employed for the complete hydrolysis of sucrose solution (5 to 40% w/v). Entraped invertase was repeatedly used (50°C, 3 h reaction) up to 30 times. As the results show little change occurred in the hydrolysis pattern of the substrate between the 1st and 30th trial (Fig. 11). Immobilized invertase was further packed in a column and 5 or 30% sucrose solution was fed continuously. The yield of hydrolyzed product increased at the higher substrate

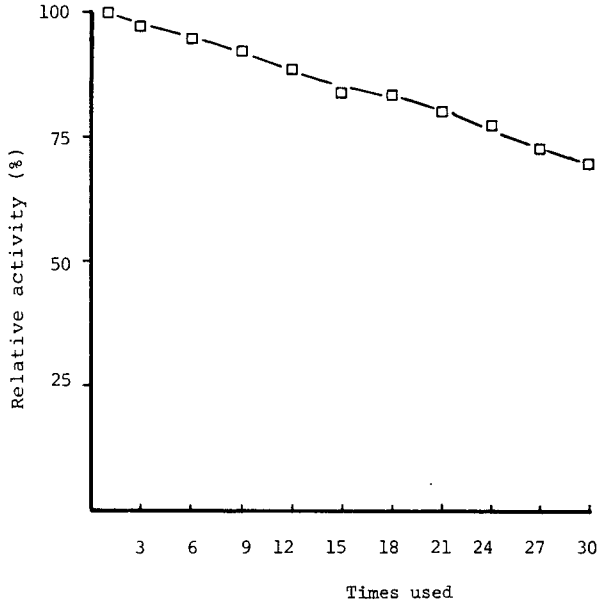

FIG. 10. Activity change of immobilized invertase when used repeatedly (29). 1/50 of I-0.5 was subjected to the enzymic reaction at 40°C for 15 min repeatedly up to 30 times. Citrate 1.1% sucrose was the substrate. Phosphate buffer pH3.5 containing I-0.5 is explained in Fig. 7.

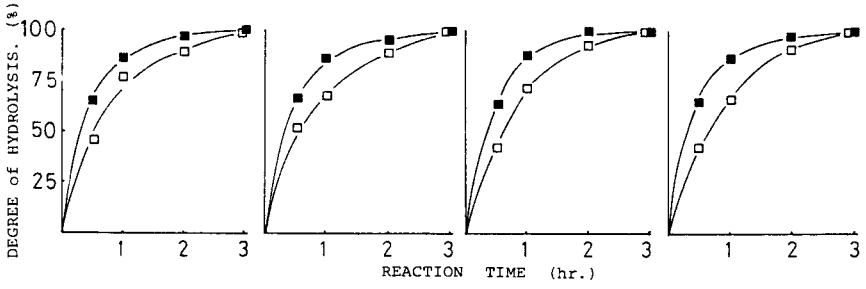

FIG. 11. Hydrolysis pattern of sucrose solution by immobilized invertase used repeatedly (31). From left to right: hydrolysis patterns of sucrose solution after 1st, 10th, 20th, and 30th usages of immobilized invertase. 100 ml of 10% sucrose (citrate-phosphate buffer pH 3.5), one (—□—) or two (—■—) membranous immobilized invertase (I-1.0, cut into small pieces). Reaction temperature, 50°C, 3 h reaction up to 30 times.

TABLE 3. **Combinations of Polymerizing Reagents for the Immobilization of Glucose Isomerase (30)**[a]

No.	30% ANa	30% ACa	30% AMg	20% P	40% P	5% PVA	10% PVA	20% PVA	HEMA	1.8% Bis	2.7% Bis	D.S.	G.I.	Sta.	1.8% Bis.	G.I.	3.6% Bis.	G.I.	Water	30% AA	glyc-erine	Retained activity
1	2 ml	2	—	—	—	—	—	—	—	—	—	0.1	1	—	—	—	—	—	—	—	—	6.1%
2	—	2	—	2	—	—	—	—	—	—	—	0.1	1	—	—	—	—	—	—	—	—	8.0
3	—	3	—	—	—	—	—	—	—	—	—	0.1	1	1	—	—	—	—	—	—	—	18.9
4	1	2	—	1	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	2.1
5	—	—	—	—	—	—	—	—	3	—	—	0.1	1	—	—	—	—	—	1	—	—	16.3
6	—	—	—	3	—	—	—	—	—	—	—	0.1	1	—	—	—	—	—	1	—	—	23.4
7	1	2	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	1	—	15.7
8	4	—	—	—	—	—	—	—	—	1	—	—	1	—	—	—	—	—	—	—	—	27.8
9	2	2	—	—	—	—	—	—	—	1	—	—	1	—	—	—	—	—	—	—	—	12.3
10	1	2	—	—	—	—	1	—	—	—	—	—	—	—	—	1	—	—	—	—	—	21.6
11	2	—	—	—	—	—	2	—	—	—	—	0.1	1	—	—	—	—	—	—	—	—	21.5
12	—	1	—	—	2	1	—	—	—	—	1	—	—	—	—	—	—	—	—	—	0.5	3.8
13	3	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	16.7
14	—	—	4	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	53.7
15	—	—	3	—	—	—	—	1	—	—	1	—	—	—	—	—	—	—	—	—	—	65.1
16	—	—	3	—	—	1	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	77.8
17	—	—	1	—	1	1	—	1	—	—	1	—	—	—	—	—	—	—	—	—	—	25.7

[a]AA, ANa, ACa are described in Figs. 1 and 2. AMg, magnesium acrylate; P, vinyl pyrrolidone; HEMA, hydroxyethyl methacrylate; Bis, *N,N'*-methylenebisacrylamide; D.S., divinyl sulfone; G.I., glucose isomerase; Sta, starch.

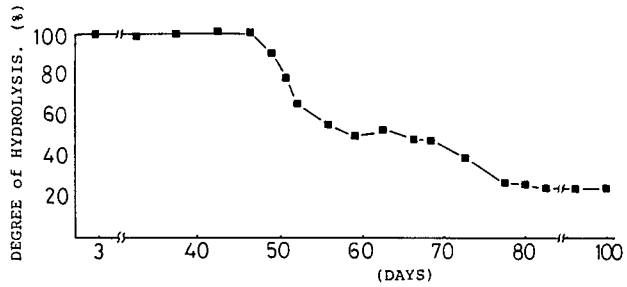

FIG. 12. Continuous hydrolysis of sucrose solution by immobilized invertase column packed with I-20(31). Thirty percent sucrose solution was fed continuously at the flow rate of SV 1 (20 ml/hr) at 50°C (citrate phosphate buffer pH 3.5).

concentration and also at higher substrate feed rates. Thirty percent (w/v) sucrose solution was inverted by the immobilized enzyme column at the flow rate of SV 1.0 over a 48-day period of operation (Fig. 12). The membranous and beaded immobilized invertase column showed similar characteristics (24).

IMMOBILIZATION OF GLUCOSE ISOMERASE

From the industrial point of view, glucose isomerase is promising and there are many reports on its immobilization. The radiopolymerization method was applied. First, the selection of suitable reagents was conducted. In Table 3, magnesium acrylate was found to be essential (Nos. 14 to 17). Glucose isomerase requires Mg^{2+} for its activity and perhaps this is the reason why magnesium acrylate was favored. The enzyme is surrounded by Mg^{2+} within the polymer. The retained activity of glucose isomerase was about 50 to 80%. After immobilization, the enzyme showed greater stability against heavy metals. There were no changes in the optimum reaction temperature and the optimum reaction pH. After shaking with 5 M NaCl for 2 h at 30°C 20 to 40% of the activity was lost, mostly due to leakage. In order to prevent the leakage, the enzyme was immobilized in the presence of glutaraldehyde or after immobilization the glucose isomerase was soaked in glutaraldehyde solution. However expected results have not been obtained so far.

IMMOBILIZATION OF OTHER ENZYMES

As the merit of entrappment method some other enzymes are also immobilized by radiopolymerization at low temperatures. These enzymes

were glucose oxidase, *d*-amino acid oxidase, acylase, catalase, lipase, urease, amylase, protease, peroxidase, pyruvate kinase, NAD kinase, and NADH oxidase.

Polymerizing reagents are inexpensive. The immobilization process is quick, simple, and no skilled technique is needed. Most of the enzymes and microorganisms can be immobilized in a bead shaped with high enzymic activities.

If a γ source is available, this method is really worth trying.

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