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Studies on the Immobilization of Enzymes and Microoganism

Part 1. Immobilizing Method of Glucose Oxidase by Gamma Radiation

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酵素 및 微生物의 固定化에 관한 研究

第1報. 放射線照射에 의한 Glucose Oxidase의 固定化法

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Abstract

A new method for immobilization of glucose oxidase by the aerobic gamma radiation of synthetic monomers was developed. The radiocopolymerization was conducted aerobically at −70 to −80°C with the mixture of several polyfunctional esters, acrylates and native enzyme.

The retained activity of immobilized glucose oxidase was about 50 to 55% when a NK 23G ester, acrylamide-bis and water mixture (1 : 1 : 2) in cold toluene treated with 450 krad of gamma radiation. The radiation dose did not influence significantly to the enzyme activity. The solvents used to prepare the beads of glucose oxidase and monomers were toluene, *n*-hexane, petoleum ether and chloroform.

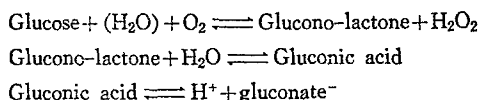
0. 05M tris-gycerol (pH 7. 0) was a more suitable buffer solution for immobilizing the enzyme than was 0. 02M phosphate. Immobilization of glucose oxidase shifted the optimum pH for its reaction from 6. 0 to 6. 5. The pH profile for the immobilized enzyme showed a broad range of optimum activity while the native enzyme gave a sharp pick for its optimum pH value. The immobilized enzyme reaction temperature was at the range of 30~40°C.

Introduction

Enzymic analysis is the most reliable way to assay for small amounts of certain components in mixture which contain various substances. However, the enzyme is usualy expensive and the number of samples to be assayed is increasing. Furth more, enzymic assay should be quickly done.

The term 'immobilized enzyme' as defined here, means the pysical confinement or localization of enzyme molecules during a continuous catalytic process. The many methods for enzyme immobilization, which had been described over the last decade, [1,2] have recently been classified into four groups. [3] These groups are (1) chemical attachement to water-insoluble support meterials, (2) adsorption in water-insoluble organic or inorganic supports, (3) entrapment within gel lattices or semipermeable microcapsuls, and (4) containment within semipermeable membrane-dependent devices.

β-D-glucopyranose aerodehydrogenase or glucose oxidase, GOD (β-D-glucose-oxygen-1 oxidoreductase, EC 1. 1. 3. 4) catalyzed the oxidation of β-D-glucose to D-glucono-δ-lactone, which subsequently hydrolyzes rapidly D-gluconic acid. The reactions are:



$$\text{Glucose} + (H_2O) + O_2 \rightleftharpoons \text{Glucono-lactone} + H_2O_2$$
$$\text{Glucono-lactone} + H_2O \rightleftharpoons \text{Gluconic acid}$$
$$\text{Gluconic acid} \rightleftharpoons H^+ + \text{gluconate}^-$$

Glucose was first determined manometically[4] by glucose oxidase and then later colorimetrically[5]. The optimum conditions for the routine determination of glucose in blood have been extensively studied[6,7]. As disaccharides are normally absent from blood, the 'true glucose' is obtained even if impure glucose oxidase preparation are used. It is necessary to use highly purified glucose oxidase for analysis of sugar mixtures.

In recent years, a considerable amount of work has been carried out on immobilized enzymes, [8,9] but relatively few studies are concerned with the immobilization of glucose oxidase.

Glucose oxidase was immobilized in polyacrylamide gel, [10] and covalently bound to nickel oxide, [11] but poor results were obtained due to the instability and the low activity of the preparation using these techniques. Enzyme gel-entrapment is usually conducted by polymerization of acrylamide. [12,13] The author has been investigating the polymerization of water soluble monomers and polymers including acrylamide, acrylates and polyvinyl alcohol by gamma irradiation. [14] It is well known that enzyme proteins show a relatively high radioresistance during the food irradiation and radiosterilization of enzymes.

It was expected that enzyme could be entrapped in the polymer matrix by radiocopolymerization of these compounds tested. To assist in the physical entrapment of enzyme proteins in filamentous structures, synthetic monomers were used to prepare water-insoluble derivatives of glucose oxidase.

This study describes the properties of the native and the immobilized glucose oxidase by radiocopoly merization. Optimal conditions for the stability and activity of the native and immobilized enzyme are reported.

Materials and Method

1. Materials

The native glucose oxidase purified from *Aspergillus niger* was provided by the National Food Research Institute, Tokyo.

o-Dianisidine was recrystallized from 25% acetone by the addition of charcoal, because the commerical product of free base *o*-dianisidine was coloured. Ten grams of the recrystallized base was dissolved in 200 m*l* of distilled water and 8. 5 m*l* of concentrated HCl 1, 000 m*l* of acetone was added. The crystals were filtered off and washed with acetone and ether, and dried in vacuum over with KOH.

2. Synthetic Monomers

The following monomers were used to prepare water-insoluble matrixs containing glucose oxidase by radiation copolymerization.

Acrylamide (AA) and *NN'*-methylene-*bis*-acryl-

amide (*Bis*) were purchased from Seikagaku Kogyo Ltd. of Tokyo. The acrylamide was recrystallized from acetone before use. Hydroxyl ethylmethacrylate (HEMA), polyvinyl alcohol (PVA) and several acrylates were used as polymerizing substances. NK ester monomer M4G, 14G, A14G, and 23G were purchased from Shin Nakamura Chemical Ltd., Japan.

3. Radio-Immobilization

Monomers were combined as shownd in Table 1. One m*l* of the native enzyme solution was mixed with 4 m*l* of each monomers solutions. Each mixture was injected into about 100 m*l* of toluene held in a 200 m*l* test tube cooled by a dry ice-acetone solution.

The injected mixture changed to small frozen beads precipitating to the bottom of the tube. The bead size could be controlled by the technique of injection and by the kind of monomers. The mixtures were irradiated with gamma rays of 60 Co at about 5,000 Ci at the dose rate of 420∼450 krad/h by Gamma Cell 220, Canada AEC. The radiocopolymerization was performed under frozen conditions.

After radio-immobilization, the tube was thawed with a buffer solution at the room temperature and the bead shaped polymers were produced by decandation of the ether. The immobilized beads of glucose oxidase were finally obtained by washing with distilled water.

4. Activity Assay

All of the hydrogen peroxide resulting from the catalytic action of glucose oxidase on glucose should oxidized the reduced form-dye by peroxidase. The amount of dye formed from the dye-H_2 is a measure of the amount of glucose oxidized. The immobilized enzyme activity on the monomer screens was determined in terms of μg of enzyme ac tivity based on the activity of a known quantity of native enzyme. The substrate for all experiments was anhydrous 5% D-glucose dissolved in 0.05 M tris buffer (pH 7.0). The substrate solution was contained 20 mg per cent of o-dianisidine and 60 μg percent of peroxidase.

The enzyme was assayed as follows. One ml aliquot containing 0.5 μg of native enzyme or 100 mg of immobilized enzyme was added to 5 m*l* of the complete substrate solution which was preincubated at 37°C for 10 min. Of cause, the enzymes should be preincubated separately. The reactants were exactly incubated with shaking at 37°C for 30 min. After the reaction, 5 m*l* of 4 N HCl was added into the reactant tube and placed it at room temperature after mixing thoroughly. The solution was examined spectrophotometically at 525 nm with the blank run by the same procedure.

Result and Discussion

1. Radiation Copolymerization

Radiation polymerization is usually performed anaerobically because oxygen inhibits polymeriza tion reactions. The polymerizations might be carry ed out by chemical catalysts and enzymes have been immobilized in the matrix preparation which was almostly polymerized with acrylamide by the chemical catalyst[16,17].

However, a method for the polymerization of acrylamide has been developed by an aerobic reation[15], and the aerobic reaction utilizing radiation technique has been substituted for chemical catalysts.

The native glucose oxidase activity and the retained activities of bead-shaped enzyme by gamma irradiation are showed in Fig. 2. The glucose oxidase aliquot dissolved with distilled water has over 90% of the activity at the dose of 215.1 krad but it decreases gradually according to increase of the dose. The bead-shaped enzyme has over 90% of the activity at 322.0 krad and it also retains 70% of the activity at 860.2 krad.

The bead-shaped enzyme has higher retained activity then water soluble enzyme and it describes that the shaped bead of acrylamide in frozon solution has remarkably protected the enzyme to ionizing radiation. Radiation effects of enzyme have been reported by changes of peptide chanins[18,19] or destory of high moleculars of protein[20,21].

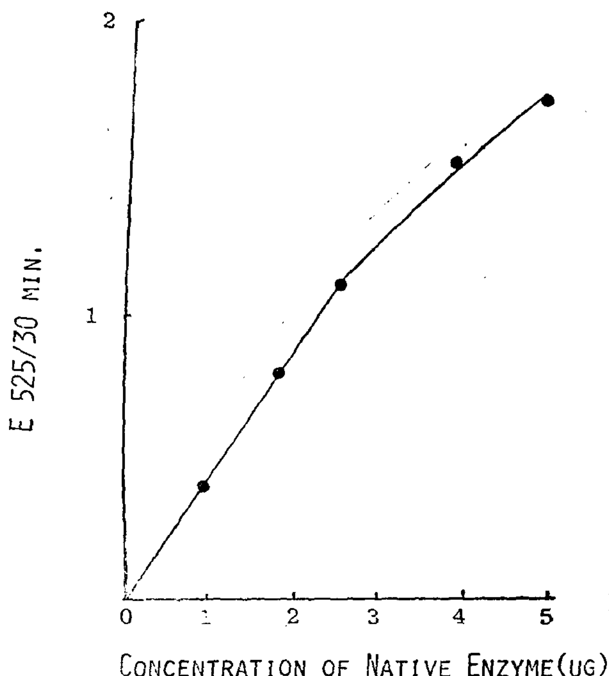


Fig. 1. Reaction Curve of Glucose Oxidase.
Various concentrations of glucose oxidase were dissolved in 1 m*l* of 0.05 M tris buffer (pH 7.0) and then added to a o-dianisidine and peroxidase mixture. The reactant was exactly incubated at 37°C Following treatment with adding 5 m*l* of 4 N HCl.

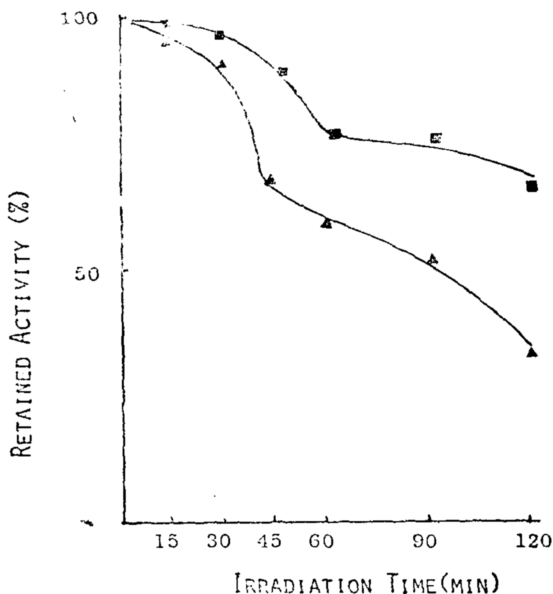


Fig. 2. Radiation Effect on the Activity of Glucose Oxidase.
(▲) native enzyme (6 μg/m*l*) and (■) beaded enzyme in frozen toluene, were irradiated with gamma ray from ^{60}Co at the dose rate of 430.2 krad/h.

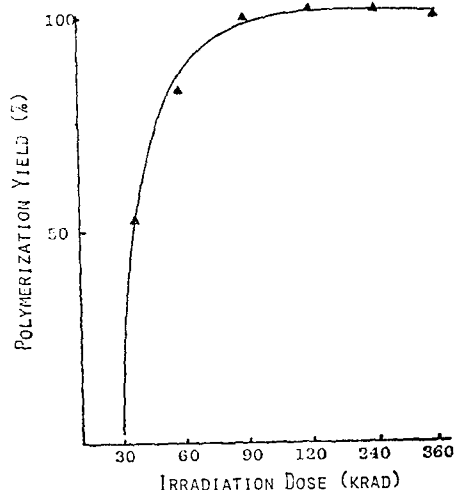


Fig. 3. Polymerization Yield from Monomers.
The polymerization yields of the monomers given in the text, were expressed as a percentage of the weight of obatined beads which dryed then vacuum oven for 3 hr, divided by the initial monomer weight.

The radiocopolymerization yield of the monomars as sample No. 18 combination in Table 1. was represented the percentage of dryed weight of the produced beads after irradiation, to the initial mo nomer weight. The monomers were polymerized about 95% at the dose of 75 krad of gamma ray and the polymerization rate was constant to increase by 360 krad.

2. Monomer Combination

It is one of the most important problems in the process of immobilizing enzyme to find out more adequate monomers and their combination in oder to obtain high retained activity of the enzyme immobilized. The various monomers combinations were prepared with 1 m*l* of the native enzyme solution and 4 m*l* of monomer solutions, and they were irradiated with 450 krad of gamma ray. One hundred some combinations have been examined in this study of glucose oxidase, and some of the interesting combinations are particularily choosed in the Table 1.

The combinations appeared the high retained activities over 40%, are sample No. 10 (42.4%) No. 17 (55.5%), No. 18 (55.8%) and No. 19

Table 1. Retained Activities of Immobilized Glucose Oxidase by Various Monomers Combinations.

Sample	Monomer (m*l*)[1]											Water	Enzyme	Retained
Number	AA-Bis	AMg	ANa	ACa	M4G	14G	A14G	M9G	23G	HEMA	PVA	(m*l*)	(50μg/m*l*)	Activity(%)
1	1		1	2									1	26.7
2	1	2	1										1	22.5
3	2	1									1		1	31.2
4					4								1	35.0
5						4							1	35.6
6	2									2			1	32.2
7		1				2				1			1	18.8
8	1			1			2						1	23.8
9		1				2					1		1	27.5
10	1				1	1	1						1	42.4
11							4						1	30.4
12								4					1	22.9
13	1				1	1	1						1	37.4
14	2						1	1					1	27.1
15							2	2					1	26.8
16									4				1	28.0
17	2								2				1	55.5
18	1								1			2	1	55.8
19	2								0.5			1.5	1	53.6
20	0.5								2			1.5	1	37.6

1 AA-Bis: 30% Acrylamide+2.7% *N, N'*-Methylen *bis*-acrylamide.
AMg : 30% Magnecium Acrylate. ANa: 30% Sodium Acrylate.
ACa : 30% Calcium Acrylate. PAV: 30% Polyvinyl Alcohol.
HEMA : 30% Hydroxyethylmethacrylate.
M4G, 14G, A14G, M9G, 23G: Naka Ester, Inhibiter MeHQ-100, Shin Nakamura Chemical.

Table 2. Effect of Chilling Media on Bead Formation and Retained Activity of Immobilized Enzyme.

Media	Formation of Bead	Activity[1)]
Acetaldehyde	−*2)	Im 7)
Acetyl acetate	±4)	Im
Acetone	±	Im
Benzene	±	Im
Butanol	−3)	Im
n-Butanol	±	Im
Carbon tetrachloride	−*	Im
Chloroform	++6)	40.48
Dioxane	+5)	34.19
Ethanol	−	Im
Ethyl ether	++	50.98
n-Hexane	++	55.08
Isoamyl alcohol	−	Im
Isopropanol	++	44.78
Methanol	±	Im
Methylcellosolve	±	Im
Petoleum ether	++	54.15
n-Propyl alcohol	−	Im
Propylene glycol	−*	Im
Pyridine	−*	Im
Tetraamyl alcohol	−*	Im
Trichloroethylene	+	33.95
Toluene	++	56.10
Xylene	++	51.04

1) Retained activity of immobilized glucose oxydase by the gamma ray of 450 krad.
2) Frozon media. 3) The mixture dissolved.
4) Massive gel formed. 5) Colonial beads.
6) Separated beads.
7) Impossible assay due to lack of bead formation.

(53.6%). They are not the combination of acrylamide and acrylates used by Kawashima *et al*, [(22)],

and it hereby should be mentioned particularily that the combinations were first applied by new developed polyfunctional monomer and AA-Bis. One m*l* of AA-Bis, 1 m*l* of 23G, and 2 m*l* of distilled water as the same the combination of sample No. 18 are available to the next works to check the properties of immobilized glucose oxidase.

3. Effects of Optimum Conditions on the Immobillization

Organic solvents to be affected to bead formation and the retained activity were listed in Table 2.

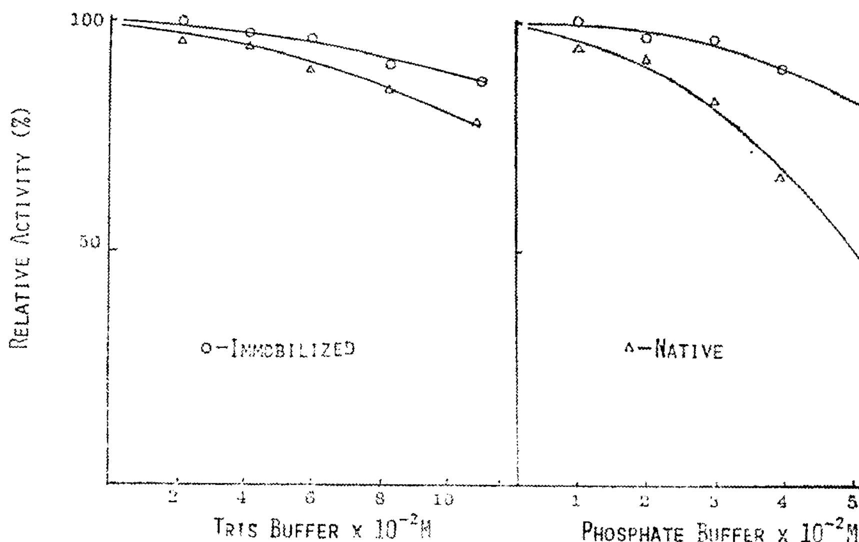


Fig. 4. Effect of Buffer Concentration on Glucose Oxidase.
The tris-glycerol buffer contained 10% glycerol and was adjusted to pH 7.0 with mono basic sodium phosphate. The phosphate buffer was prepared with mono and di-basic sodium phosphate to pH 7.0.

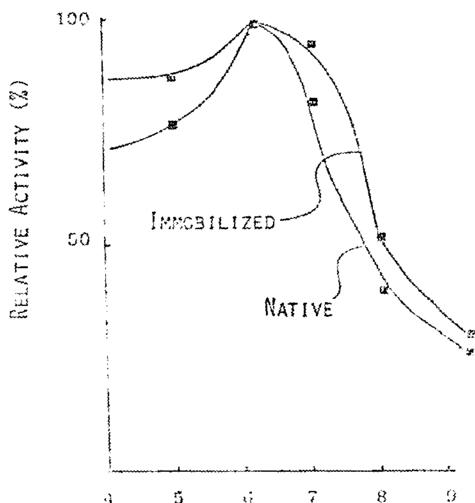


Fig. 5. Effect of Substrate pH on Glucose Oxidase.
GOD was immobilized by adding 5 μg/m*l* of the native enzyme to 4 m*l* of monomer solutions.
The native and immobilized enzymes were assayed at various pH for 30 min at 37°C. (pH 4-6: citrate buffer, pH 7~9: tris buffer).

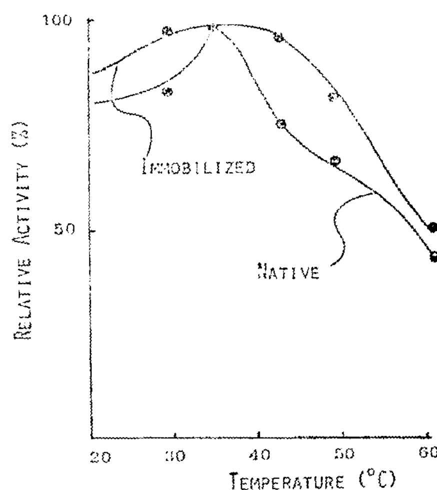


Fig. 6. Temperature Activity Profiles of Glucose Oxidase.
The native and immobilized GOD were tested at various temperature with 0.05 M tris buffer (pH 7) for 30 min. 0.5 μg of the native GOD and 100 mg of immobilized GOD were applied to each of the assay mixures.

To prohibite inactivation of enzyme in the process of immobilization, the mixture was placed with bead shaped in cooled media at -80°C.

The useful solvents for the above menthioned monomer combination are chloroform, ethyl ether, n-hexane, isopropanol, petoleum ether, toluene and xylene. Another solvents almost dissolved the mixture or were not suitable to physical attractive forms and they were difficult to assay the activity.

Glucose oxidase usually was known that it was assayed by 0.1~0.2 M-phosphate buffer (pH 6~7). Tris-glycerol buffer was compared with the phosphate buffer. The native enzyme still was stable according to the concentration of both buffer solutions, but the immobilized enzyme was sensitive to the high concentration of phosphate buffer. Tris-glycerol buffer seemed to be protected the enzyme during the immobilizing process.

The effect of substrate pH on the native glucose oxidase and immobilized enzyme is showed in Fig. 5. Both of glucose oxidase were showed similarly changes on teh different pH. However, relatively maximum activity of the immobilized enzyme was obsorved at pH 6.0~6.5 and decreased slowly on both the acid and basic side of the optimum. The native one gave maximal activity also at pH 6.0 and the curve moved more steep. The little different curves between both enzymes suggests displacement of the optimum pH and it is supposed by interaction between the surface of the sponged lattices or the microenvironment of the enzyme.

As can be seen in Fig. 6, optimum temperature profiles of the reaction of the immobilized and native enzymes are similarity.

The immobilized enzyme had the more wide range of maximal temperature as shown 30~40°C, however, the native enzyme was appeared the maximal temperature at 35°C with the sharp top of the curve..

要 約

血液 및 生體反應物質등에 含有된 微量의 glucose를 酵素的으로 간편하게 定量 하기 위하여 먼저 高價의 glucose oxidase를 固定化 시켰다.

電離 放射線照射에 의하여 이 酵素를 쉽게 固定化 시킬수 있는 方法과 그 알맞는 條件 및 높은 殘餘 活性에 관한 結果는 다음과 같다.

1) 여러가지 monomer 및 polymer의 combination 중에서 AA-Bis, NK ester 23G, 물 (1:1:2)에 可溶性 酵素 1 ml를 固定化 시켰을 경우를 비롯하여 GOD의 殘餘活性이 50%이상인 여러 monomer Combination 찾았다.

2) Carrier의 放射線重合에 필요한 線量은 100 krad 이상 이였으나, 400~500 krad가 적당하였고, solvent는 toluene, *n*-haxane, petoleum ether chloroform 등을 利用할 때 固定化된 GOD의 殘餘 活性 및 理化學的 性狀이 좋았다.

3) GOD 固定化에 利用될 수 있는 완충 용액은 tris-glycerol buffer (pH 7.0)가 phosphate (pH 7.0)보다 높은 活性을 보여 주었다.

4) 固定化된 GOD의 最適 pH는 6.0~6.5 또 溫度는 30~40°C로서 可溶性 酵素보다 作用範圍가 넓었고, pH 및 溫度의 變化도 완만였다.

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