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Design and Synthesis of Devices Releasing Insulin in response to Redox Reaction of Glucose

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Glucose의 Redox 반응에 의한 인슐린 방출 Device의 설계와 합성

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Abstract : New insulin-releasing system on the basis of the redox reaction of glucose was synthesized by immobilizing insulin through a disulfide bond(5, 5′-dithiobis(2-nitrobenzoic acid) to polymer membrane(poly(methyl methacrylate)) and enzyme(glucose oxidase). The disulfide bonds were cleaved upon oxidation of glucose with glucose dehydrogenase and glucose oxidase, releasing insulin from the membrane and enzyme. Sensitivity to glucose concentration was enhanced by coimmobilization of enzyme cofactors(nicotinamide adenin dinucleotide and flavin adenin dinucleotide) acting as electron mediator(for the membrane device), and further enhanced by direct immobilization of insulin on glucose oxidase(for the protein device). Both systems were specific to glucose, and the released insulin was indistinguishable from native insulin. The biological activity of released insulin was 81% of native insulin.

요 약

Glucose의 redox 반응에 의한 새로운 인슐린 방출계를 5, 5′-dithiobis(2-nitrobcnzoic acid)의 disulfide 결합을 이용해 인슐린을 pmma 막과 glucose oxidase에 고정화시켜 합성하였다. glucose와 glucose dehydrogenase 및 glucose oxidase와의 산화반응에 의해 disulfide 결합이 파괴되어 막과 효소로부터 인슐린이 방출된다. enzyme cofact들(nicotinamide adenin dinucleotide와 flavin adenin dinucleotide)을 coimmobilization시켜 membrane device에 대해 electron mediator로 작용하도록 하여 glucose의 농도 민감성을 향상시켰고 protein device에 대해서는 glucose oxidase에 인슐린을 직접 고정화시켜 민감성을 더욱 향상시켰다. 이 두 가지 계들은 glucose 특이성을 나타내며 방출된 인슐린은 생체인슐린과 구분되지 않았다. 방출인슐린의 생리활성은 생체인슐린의 81 % 였다.

1. INTRODUCTION

Recently, various kinds of glucose-sensitive insulin-releasing system have been designed and synthesized [1~5]. We have also succeeded in synthesizing two new systems of this type. In one of them[6, 7] a pH-responsive porous cellulosic membrane was employed, on which poly(acrylic acid) and glucose oxidase (GOD) were immobilized. When glucose is present, GOD catalyzed the conversion of glucose to gluconic acid, thus leading to the decrease of pH level. The pH change induces the conformational change of grafted polymer chain and alters the size of pores. According to this molecular mechanism, the release of insulin is controlled. The other system employed the oxidation reaction of glucose catalyzed by glucose dehydrogenase(GDH)[8, 11]. First, a composite membrane was synthesized, on which insulin was immobilized through a disulfide linkage. When glucose is oxidized by GDH in solution, an electron is transferred to the disulfide bond and cleaves it to release insulin. It was demonstrated that the coenzymes Nicotinamide Adenin Dinucleotide (NAD) and Flavin Adenin Dinucleotide (FAD) were useful to enhance the electron

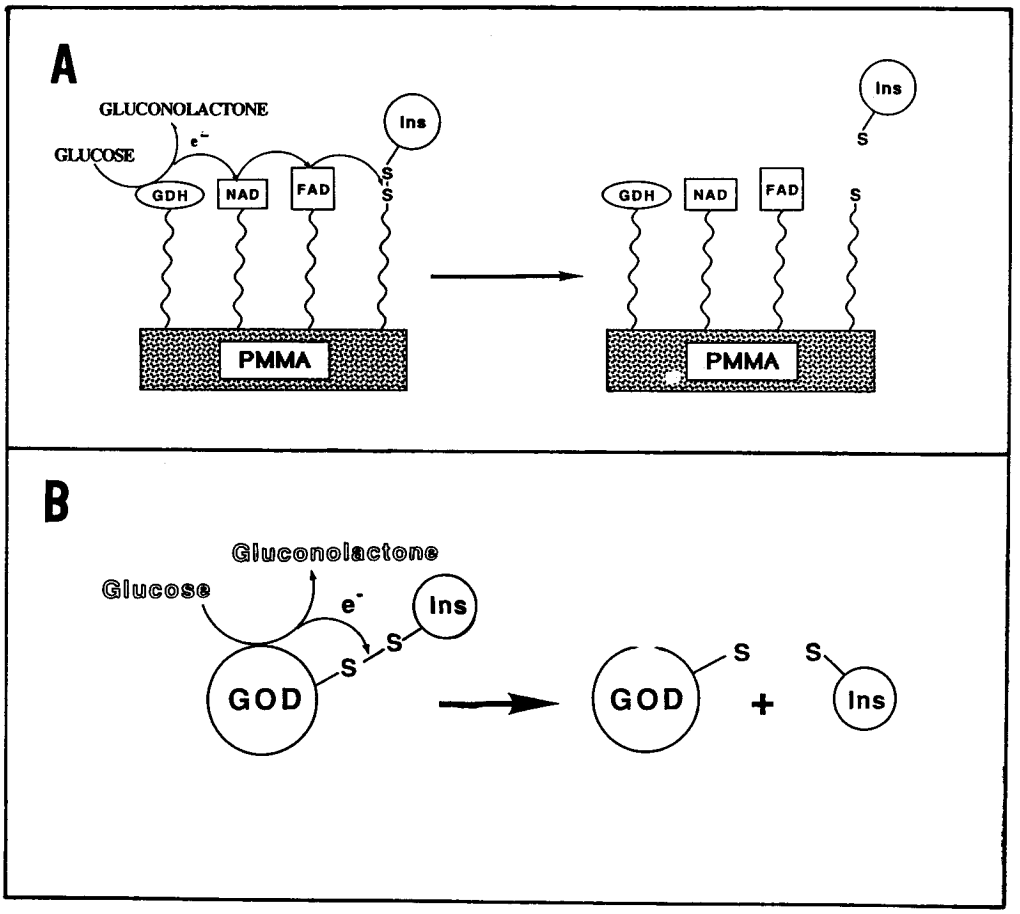

Fig. 1. Design of glucose-sensitive insulin-releasing devices. A) Membrane device, B) Protein device ; ~~~, poly(acrylic acid) graft ; Ins, insulin ; S-S, disulfide bond of DTNB ; GDH, glucose dehydrogenase ; GOD, glucose oxidase.

transfer from the GDH to the disulfide bond. Therefore in later stage, a membrane device was elaborated on which the coenzymes were also coimmobilized. It occurred to us that the reduction of the distance between the active site of the enzyme and the disulfide bond should increase the efficiency of the device. In this paper, first, the GDH was coimmobilized with the coenzyme and insulin on the membrane. Secondly a protein deivce, in which glucose oxidase was coupled directly with insulin through a disulfide bond, was synthesized. The principle of these devices are illustrated in Fig. 1.

2. EXPERIMENTAL

2. 1. Materials

Glucose dehydrogenase(GDH, derived from Bacillus sp., 56.8 I.U./mg, EC 1.1.1.47) was purchased from Amano Pharm. (Japan). Insulin(1-5500, 24.4I.U./mg of protein, derived from bovin pancreas) and Glucose oxidase(GOD, derived from Aspergillus niger, G-8135 type X, 128I.U./mg, EC 1.1.3.4) were purchased from Sigma(U.S.A.). Nicotinamide adenine dinucleotide (NAD), 5, 5′-dithiobis(2-nitrobenzoic acid)(DTNB), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride(WSC), β-D(+)-glucose and 1, 2, 3-triketohydrindene monohydrate(ninhydrin), 4-bromomethyl-7-methoxy coumarin(BrMmc), urea were purchased from Nacalai Tesque, Inc. (Japan). 2, 2′-Dihydroxy-2, 2′-biindan-1, 1′, 3, 3′-tetrone(hydrindantin), 2-methoxy-ethanol, methyl methacrylate, acrylic acid, flavin adenine dinucleotide(FAD), 2, 2′-azobisisobutyronitrile(AIBN) were purchased from Wako Pure Chem. Ind. (Japan). Ethylenediamine(ED) was purchased from Tokyo Kasei Ind. (Japan). Ninhydrin-hydrindantin solution was prepared as follows. Ninhydrin(400 mg) and hydrindantin(60mg) were dissolved in 2-methoxyethanol(15mℓ), and diluted with acetic acid-buffered solution(pH 5.5) to a total volume of 20mℓ. Acrylic acid and methyl methacrylate were distilled under a reduced pressure prior to polymerization.

2. 2. Synthesis of membrane system

The synthetic scheme is shown in Fig. 2. The immobilization to polymer membrane was carried out using WSC as reported previously[9, 10].

(**STEP 1**) Methyl methacrylate(95mℓ) in toluene (140mℓ) was polymerized with AIBN as an initiator at 45 °C for 48h. Poly(methyl methacrylate)(PMMA) was precipitated with methanol. The precipitation was repeated by using toluene and methanol. The purified PMMA was cast into a membrane(diameter 15mm) from a toluene solution(15wt %). Plasma-mediated polymerization was carried out using an Eiko IB-3 ion coater as reported previously[11]. The amount of grafted poly(acrylic acid) was determined by measuring absorbance increment at 151nm using rhodamine 6G(10wt % in benzene solution).

(**STEP 2**) When GDH was immobilized to the membrane, the poly(acrylic acid)-grafted PMMA membrane was immersed in a phosphate-buffered saline (PBS, NA_2HPO_4, 1.15g ; KH_2PO_4, 0.2g ; NaCl, 8.0g ; KCl, 0.2g ; $CaCl_2$, 0.1g ; $MgCl_2$, 0.1g in distilled water, 1l) containing WSC(10mg/mℓ) for 2h at 4 °C. After washing with PBS, the activated membrane was immersed in a PBS containing GDH(62.9 I.U./mℓ) for 20h at 4 °C.

(**STEP 3**) The GDH-immobilized membrane was immersed in a PBS containing WSC(10mg/mℓ) for 2h at 4 °C. After washing with PBS, the activated membrane was immersed in a PBS containing NAD, FAD, and ED of known concentrations which were variable.

(**STEP 4**) The enzyme/coenzyme-immobilized membrane was immersed in a PBS containing WSC (10mg/mℓ) and DTNB(10mM) at 4 °C for 20h.

(**STEP 5**) The DTNB-immobilized membrane was immersed in a PBS containing WSC(10mg/mℓ) for 2h at 4 °C. After washing with PBS, the activated membrane was immersed in a PBS containing insulin (61 I.U./mℓ). After each immobilization procedure. the membrane was repeatedly washed with PBS until fed substances were not detected in the washing solution by ultraviolet measurement.

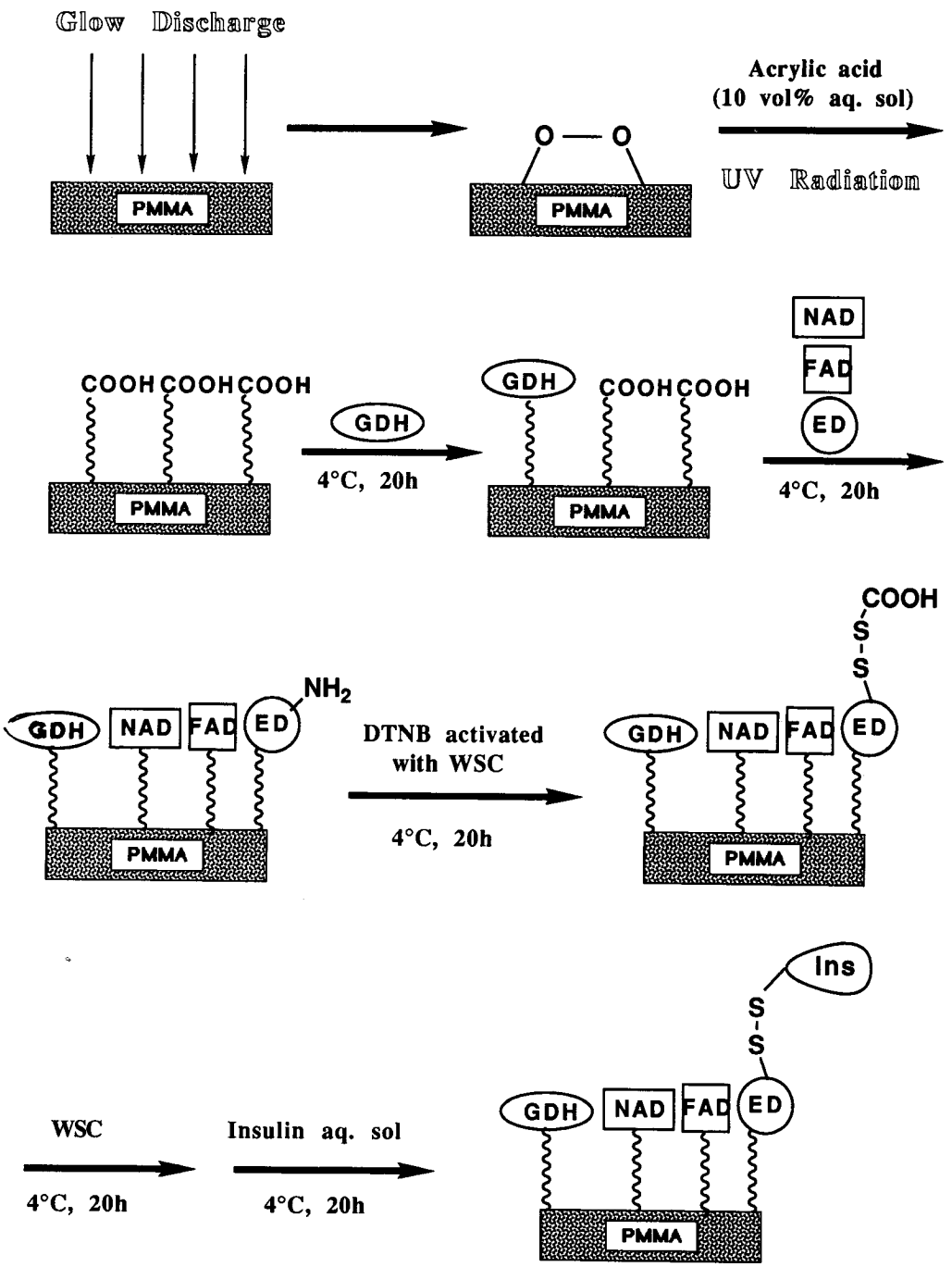

Fig. 2. Synthetic scheme of the GDH/coenzymes/insulin-immobilized polymer membrane. ED, ethylenediamine ; WSC, water soluble carbodiimide ; UV irradiation at 0 for 2h.

2. 3. Synthesis of protein device system

The synthetic scheme of the protein deivce is shown in Fig. 3. Fluorescent labelling reagent(BrMmc, 50mg(0.18mmole)) was coupled to insulin(100mg) in acetone solution(100mℓ) with K_2CO_3(312mg, 2.26 mmole) by refluxing for 1hr in order to block the COOH groups of the insulin, and to prevent the formation of amide bond between COOH in insulin and NH_2 in GOD. After centrifugation and passing through Sephadex G-15 GPC column, labeled insulin was separated. And then, coupling of DTNB to labeled insulin(100mg) was performed in the presence of WSC (638mg, 3.3mmole) in PBS(15mℓ) at 4 °C for 20h by changing the feeding molar ratio of DTNB. The purification procedure was carried out Sephadex G-15 GPC column. Finally, modified insulin with DTNB(40mg) was introduced to GOD(50mg) in the presence of WSC (300mg, 1.57mmole) and 2M urea at the same conditions. After the dialysis and lyophilization, the protein device was obtained.

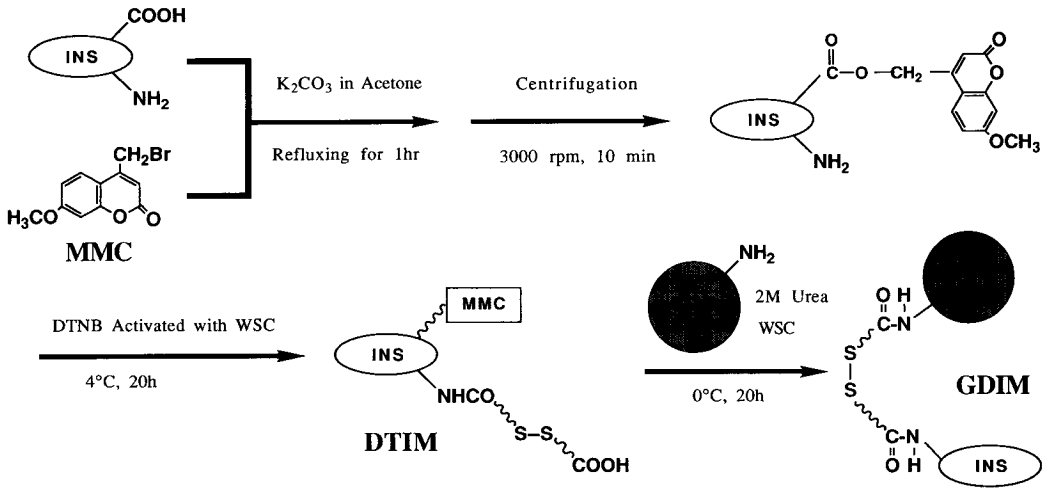

Fig. 3. Synthetic scheme of the insulin immobilized protein device. Ins, insulin ; MMC, 4-bromomethyl-7-methoxy coumarin ; DTIM, insulin modified with MMC and DTNB ; GDIM, DTIM immobilized GOD(protein device).

2. 4. Determination of immobilized substances

The amount of immobilized substance in membrane device was determined on the basis of amino compound produced in the hydrolysis of the composite membrane(GDH after the step 2 ; the total of GDH, NAD, FAD and ED after the step 3 ; the total of GDH, NAD, FAD, ED and insulin after the step 5) as reported previously[12, 13]. Poly(acrylic acid)-grafted PMMA membrane was treated in the same manner, and the absence of amino compound generation was confirmed. The amounts of immobilized FAD and NAD were determined by fluorometry on Hitachi F-3010 fluorescence spectrometer(λ_{ex} (FAD), 355nm ; λ_{ex} (NAD), 286nm) after dissolving the membrane in N, N-dimethylformamide. The amount of immobilized Ed was calculated by subtraction of the amount of FAD and NAD from the total amount of immobilized substances which was determined by the previous method. The amount of immobilized insulin in protein device was determined on the basis of the S content by elemental analysis of native GOD, insulin, modified insulin with DTNB and protein device.

2. 5. Release experiment

Release experiment was performed as follows. Insulin-immobilized membranes(six sheets, for the membrane device) or synthesized protein device(10mg)

were kept at 37 °C for 30min in PBS, and a glucose solution of a prescribed concentration(1.4㎖) was added to the mixture at constant time intervals. Absorbance of the supernatant solution at 280nm or fluorescent intensity was measured with one minute intervals at each case. Released insulin was analyzed by a high-pressure liquid chromatography using a Cosmosil 5Diol-120GPC column(pressure, 50kgf/cm^2 ; solvent, 1M Na_2HPO_4 ; rate, 1㎖/min). The measurements were repeated three times and the obtained values were averaged.

2. 6. Measurement of biological activity

The activity was measured by the method reported previously[14]. Mouse fat cells were pooled, washed 4 times and diluted in Krebs-Ringer bicarbonate buffer containing 0.55mM glucose and 10mg of albumin/㎖. One ㎖ aliquot of the suspension were added to flasks containing PBS solution of ^{14}C-labelled glucose(0.1μCi, 20μℓ) and PBS solution of insulin derivatives(20μℓ). The cells were incubated at 37 °C in 20㎖ scintillation flasks for 2h, and the incubation was terminated by the addition of 8 N H_2SO_4(200μℓ) and then added to a toluene scintillator(15㎖). The activity of native insulin was taken as the standard.

3. Results and Dicussion

3. 1. Membrane device

The amount of immobilized species was determined and is shown in table 1. The amount of FAD and NAD immobilized on the membrane was smaller than that in the absence of the GDH which was reported previously[11]. This decrease in immobilization amount could be attributed to the bulkiness of the pre-immobilized GDH. But, the amount of ED was slightly changed, therefore the amount of immobilized insulin was almost same as that of previously reported.

Table 1. The Amount of Substances Immobilized on the Poly(Acrylic Acid)-Grafted Poly(Methyl Methacrylate) Membrane

Molar ratio of FAD/NAD/ED/ in feed[a)]	Amount of GDH immobilized on PMMA membrane (μg / cm^2)	Amount of NAD, FAD and ED immobilized on PMMA membrane (x 10^{-9} mole / cm^2)			Amount of insulin immobilized of PMMA membrane (x 10^{-9} mole / cm^2)
		FAD	NAD	ED	
1/ 1/ 1	0.11 ± 0.02	0.04± 0.01	0.07± 0.03	1.62± 0.60	0.030± 0.006

a) The concentration of FAD was always 0.5 mg/㎖

Fig. 4 shows the insulin release in response to glucose concentration. A large amount of insulin was quickly released in response to glucose of a low concentration(9.8mM), though a negligible amount of insulin was released in response to glucose of a lower concentration(0.98mM). 95 % of immobilized insulin was ultimately released in response to added glucose. It is known that the glucose concentration in human blood is in the range from 5 to 15mM, and that the insulin concentration varies from 0.13 to 0.6×10^{-12} mol/㎖ according to the level of glucose concentration. Fig. 4 shows that 2.85×10^{-11}mol/㎖, 0.17μg/cm^2, of insulin was released in response to 9.8mM glucose. The moles of released insulin per mole of added glucose in this system were relatively high, as compared with that occurring in the living body. This result indicates that coimmobilization of GDH with electron mediators facilitates the electron transfer on the membrane

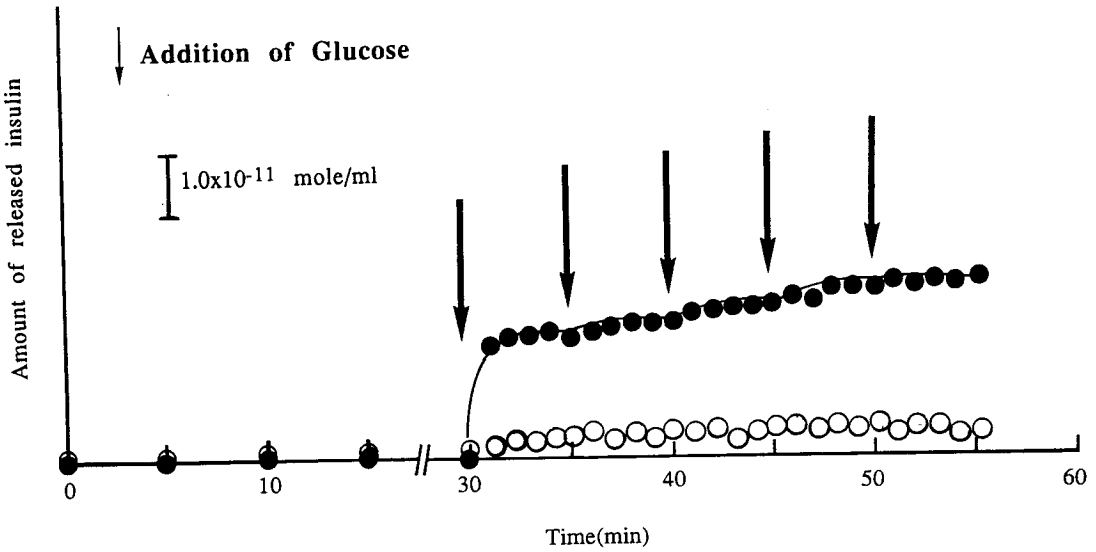

Fig. 4. Release of insulin from a composite membrane prepared with NAD/FAD/ED molar ratio in the feed of 1/1/1 with GDH coimmobilization. Concentration of added glucose, 9.8mM(●) and 0.98mM(○) in PBS.

3. 2. Protein device

In order to reduce the distance between the active site of enzyme and the disulfide bond, insulin was directly connected to enzyme(GOD). The amount of immobilized insulin was shown in Table 2. As the molar feeding ratio of modified insulin to GOD increases, the amount of immobilized insulin on protein device also enhances. To compare with the effect of presence or absence of urea in the synthesizing step of protein device, the amount of insulin immobilization was investigated under same molar feeding ratio. As a result of urea addition, the amount of immobilized insulin was increased. It can be inferred from this result that internal NH_2 groups of enzyme are liable to generate amide linkage against the uncoupled COOH group in DTIM according to the conformational change of enzyme by adding urea.

Table 2. The Amount of Insulin Derivatives Modified with DTNB Immobilized on the Protein Device

Molar ratio of DTNB/Insulin in feed	Molar ratio of modified insulin/ GOD in feed	Urea (2M)	Content of S (%)	Amount of immobilized insulin(mole %)
5/1	10/1	+	3.39	38
	1/1	+	2.27	20
		−	1.62	11
	1/10	+	1.67	11

In Fig. 5, fluorescence spectra of labeled insulin and protein device were shown. The fluorescence attributed to BrMmc was appeared at 400nm in labeled insulin and protein device(curve B and C). But there was no fluorescence in native insulin.

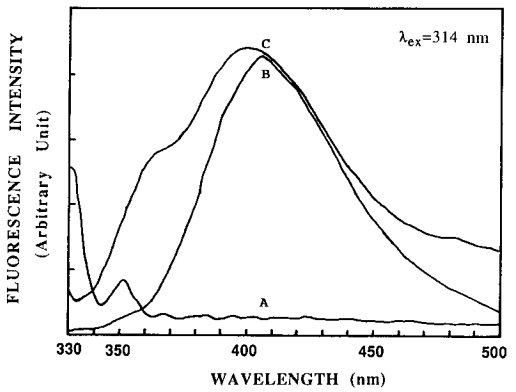

Fig. 5. Fluorescence spectra of insulin derivatives and protein device in PBS at room temperature. A, native insulin ; B, insulin modified with MMC and DTNB ; C, protein device, λ_{ex}=314nm.

The insulin releasing pattern of the protein device after the glucose addition(0.98mM) was shown in Fig. 6. Before addition of 0.98mM glucose, no insulin release was detected for two kinds of protein device. Insulin release could be detected in protein device by addition of glucose and this tendency continued after many times of glucose addition at the same condition, though insulin release was not occurred in the membrane device by the addition of 0.98mM glucose. The amount of released insulin was increased with the amount of immobilized insulin.

The biological activity of released insulin was assessed by the glucose uptake method using fat cell. The activity was 81 % as high as that of native insulin. A similar activity of modified insulin to that of native insulin has been reported with glycosylated insulin [15].

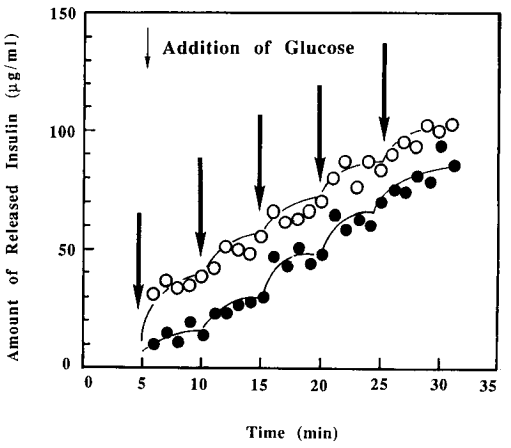

Fig. 6. Release of insulin from a protein device prepared with DTIM/GOD molar ratio in the feed of 1/1(+)(○) and 1/10(●). Concentration of added glucose, 0.98mM in PBS.

4. CONCLUSION

New types of glucose-sensitive insulin-releasing systems were designed and synthesized. The most efficient system consists of immobilized insulin and enzyme(GOD) through a disulfide bond. It was shown that insulin was released by reductive cleavage of the disulfide bond, that the insulin releasing system was specific to glucose, and that disulfide bonds involved in the released insulin were kept intact during a series of reactions.

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