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Controlled Release of Insulin through Glucose Oxidase Immobilized Composite Poly(vinyl Alcohol)/Chitosan Blend Membrane

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글루코오즈가 고정화된 Poly(vinly Alcohol)/Chitosan 블렌드 복합막을 통한 인슐린의 방출조절

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Abstract: The permeation of insulin was conducted through glucose oxidase(GOD) immobilized composite membrane composed of poly(vinyl alcohol)/chitosan blend and porous polyamide membrane. The permeation coefficient of insulin through GOD-immobilized membrane was in the order of 10^{-6}~10^{-7}cm^{3}cm/cm^{2}sec. The sensitivity of the composite membrane to the glucose concentration was high in a low glucose concentration resulting from the oxygen depletion from the membrane. The permeation of insulin through composite membrane made of PVA/chitosan and porous polyamide membrane was changed by pH and glucose concentration. The permeability was progressively increasing with the glucose concentration at least up to 500mg%.

요 약: 글루코오즈(GOD) 옥시다제가 고정화된 PVA/키토산 블렌드막과 다공성 폴리아미드 복합막을 통해 인슐린의 투과거동을 살펴보았다. GOD가 고정화된 막을 통한 투과계수는 10^{-6}~10^{-7}cm^{3}cm/cm^{2}sec이었다. 복합막의 글루코오즈 농도에 대한 변화는 낮은 글루코오즈 농도에서 높았는데 이는 막으로부터 산소의 고갈 때문이었다. PVA/키토산 및 다공성 폴리아미드막을 통한 인슐린의 투과는 글루코오즈 농도에 따라 500mg%까지 점차 증가하였다.

1. Introduction

Diabetics mellitus(D. M) is an international disease affecting 1~2% of the population in most countries of the world, especially in the advanced countries which are not afflicted by malarial, biharzia, tuberculosis and gastrointestinal diseases [1]. Diabertics is a chronic complex metabolic dis-

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ease. Controlled release of insulin is useful for the treatment of diabetics because the conventional insulin adminstr-ation by injection causes a wide fluctuation in blood glucose level. Many studies on the controlled release of insulin using polymeric materials have been reported[2~4]. For an ideal insulin delivery system, insulin release should be controlled directly by the amount of blood glucose present at any particular time. This requires a continuous feedback of the glucose level in the blood and insulin release rate[5~7].

We have already reported riboflavin and insulin permeation through poly(vinyl alcohol) (PVA) and chitosan blend membrane in response to pH and glucose concentration[8]. It was noted that the higher glucose concentration and thus a lower pH led to a more swollen membrane. A decrease in pH is attributed to the enzymatic reaction between GOD and glucose to form gluconic acid. The increase in water content in the PVA/chitosan blend membrane is due to the protonation of amino groups in the membrane in response to a decrease in pH. Therefore, the water content and thus the insulin permeation of PVA/chitosan blend membrane can be changed by the enzymatic reaction between GOD and glucose. However, the response of GOD in PVA/chitosan blend membrane to glucose showed that the pH decrease was limited by O_2 concentration in the solution. The oxygen concentration in the blood was known to be about 2.2mol/ℓ [9]. Blood oxygen will oxidize only 40% of glucose in the normal human blood under these dissolved oxygen environment[10~11]. The consequence of this mismatch is an oxygen limitation to the range of any device which uses glucose oxidase to sense glucose. An important point in the developement of the glucose sensitive membrane as an insulin delivery system is to design a device to overcome the oxygen depletion limit. This study aims to develope the membrane controlling the glucose permeation through the composite membrane consisting of GOD-immobilized polymer membrane and the porous polyamide membrane.

2. Experimental

2. 1. Materials

Chitosan, whose degree of deacetylation was calculated to be 76% from the amino content, was purchased from Tokyo Kasei Co.(Japan), and was used after passage through 200 mesh sieve. PVA was purchased from Kuraray Co.(Japan). The degree of polymerization of PVA was 1570±50 and the saponification degree was 98.5%. Glutaraldehyde was purchased from Kokusan Chemical Work (Japan). Riboflavin was purchased from Junsei Chemical Co., LTD. Insulin from bovin pancreas (241. IU/mg) and glucose oxidase type Ⅶ from *Aspergillus niger* (125,000 U/g) were obtained from Sigma Chemical Company. Porous polyamide membrane was purchased from Gelman Science and its pore size was 0.2μm.

2. 2. Preparation of PVA/Chitosan (PCB) Membrane

Casting solution was prepared by blending PVA and chitosan (1.5 by wt.%). PVA(1.5g) was first dissolved in 5mℓ of deionized water at 90℃. One gram of chitosan was added into the PVA solution. After 30 minutes, 50mℓ of 1.5wt.% aqueous acetic acid solution was poured into the PVA/chitosan solution. Membrane was prepared by pouring casting solution onto a rimmed acryl plate and allowing the water to evaporate at 40℃ in a convective oven for a day. After immersing the membrane in 1*N* NaOH solution for a day, it was washed repeatedly with water and kept in deionized water(25℃). The thickness of the membrane used in this work was 100± 10μm. The membrane was dense in structure as seen in scanning electron microscopic pictures(not shown here).

2. 3. Preparation of GOD-Immobilized (GPCB) Membrane

PVA/chitosan blend membrane was reacted with glutaraldehyde in phosphate buffer solution(pH 7.4) in the ultrafiltration apparatus(Amicon UF Cell)

for 1.5hr. The membrane was washed five times with the buffer solution to remove any residual glutaraldehyde. The membrane was then reacted with GOD in phosphate buffer solution(pH 7.4) for one hr at room temperature and 23 hrs at 4℃. The membrane was washed with buffer solution containing 1*M* NaCl to remove non-bounded residual enzyme. The amount of enzyme bounded on the surface of the membrane was determined from the difference in concentration of GOD solution before and after the reaction. The GOD was dyed with Coomassie Briant Blue 6G(Sigma Chemical Co., U. S.A.). To prepare the solution, Coomassie Brilliant Blue 6G(100mg) was dissolved in 50mℓ 95% ethanol. To this solution, 100mℓ of 85%(w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 liter. The concentrations of dye in the standard solution and GOD solution were measured by the UV absorbance at 550nm, using spectrophotometer (Spectronic 21, Milton Roy Company, U.S.A.). Since Coomassie Briant Blue method[12] usually detects in the concentration range from 0.5~50μg, it was possible to estimate 10~100μg of enzyme concentration in our experiment. The amount of immobilized enzyme was estimated from the following equation: immobilized ratio(%)$=(C_{g0}-C_{gt})/C_{g0}\times 100$, where C_{g0} and C_{gt} are the concentrations of GOD in the UF cell measured at time $t=0$ and $t=t$, respectively. The preparation and sample designation of the membranes are given in Table 1. Finally a 10wt% $NaHSO_3$ solution was added to deactivate the unreacted aldehyde groups on the PVA/chitosan blend membrane.

Table 1. Preparation of Gluose Oxidase Immobilized PVA/Chitosan Blend Membrane

Sample Name	Glutaraldehyde ($\times 10^{-6}$mol/cc)	Glucose oxidase (g/100cc)
B-1-1	12	0.1
B-1-2	12	0.05
B-1-3	12	0.01
B-2-1	6	0.1
B-2-2	6	0.05
B-2-3	6	0.01
B-3-1	3	0.1
B-3-2	3	0.05
B-3-3	3	0.01

PVA(1.5g) and Chitosan(1.0g)

2. 4. Degree of Swelling

The weight of completely dried sample was measured directly, and the sample was dipped into the petri dish filled with a different pH phosphate buffer solution where temperature was measured at 37 ℃ in an incubator. Also GOD-immobilized membrane was palced in petri dishes at different glucose concentration and kept in an oxygen atomosphere. The weight of swollen membrane was measured after 24hr. The degree of swelling of these samples was calculated with the following equation: degree of swelling$(Q_w)=(W_2-W_1)/W_1\times 100$ where W_1 and W_2 are the weight of dry and swollen samples measured at a different time period, respectively.

2. 5. Permeation Experiments

A permeation cell used in the present study is shown in Fig. 1 of the reference 8. It has two compartments of equal volume(100mℓ). Each chamber was magnetically stirred at 750rpm to eliminate the boundary layer resistance. All measurements were made at 37℃ in this study. One compartment of the cell was filled with a different pH phosphate buffer or with glucose solution in phosphate buffer at pH 7.4, and the otherside with a solution of riboflavin or insulin in phosphate buffer at pH 7.4. Aliquots of the buffer solution were taken out after a given period of time. During the experiment, the permeation cell was bubbled with O_2 to saturation. The UV absorbance of solution was measured with a spectrophotometer(Spectronic 21, Milton Roy Company) at 444nm in wavelength to determine the concentration of ribolflavin in the feed and in the permeate. The concentration of insulin in the feed and in the permeate was measured from the UV absorbance appeared at 274nm. At least five specimens of the samples were measured to determine the concentration. Standard deviation of the mean concentration was within ±5%. The solute

permeability coefficents P was calculated from the following equation which was obtained from mass balane equation[13], i.e.,

$$P = \frac{-d}{A(1/V_1+1/V_2)t} \ln (1 + \frac{V_1}{V_2}) \frac{C_t V_1}{C_0 V_2} \quad (1)$$

where V_1, V_2, A, d, C_0 and C_t were the volumes of the concentration and the dilute compartment, membrane area(7.96cm^2), the thickness and the concentrations of the concentrated compartment at time t=0 and t=t, respectively.

3. Results and discussion

3. 1. Enzyme Immobilization

To control the permeation of insulin through PVA/chitosan blend membrane in response to glucose concentration, glucose oxidase were immobilized on the surface of the membrane. Glucose sensitive membrane could control insulin delivery from a reservoir containing a saturated insulin solution. This type of system is the membrane containing amino group and immobilized glucose oxidase. Diffusion of glucose into the membrane and its conversion to an acid cause a pH drop and consequent increased protonation of the amino groups. The water content in the membrane increased with the glucose concentration, resulting in an increase in the permeability of the membrane.

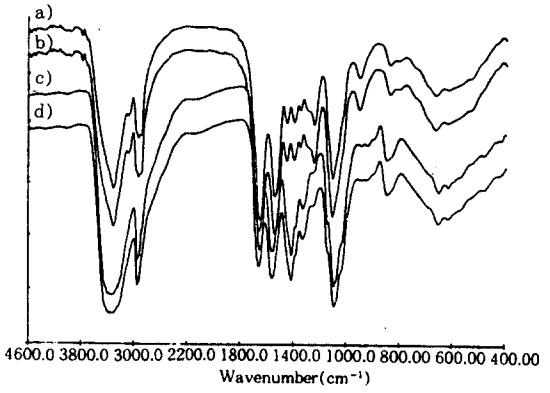


Fig. 1. a) FT-IR spcetrum of glucose oxidase, b) ATR/FT-IR spectrum of Immobilized PVA/chitosan blend membrane, c) FT-IR spectrum of immobilized PVA/chitosan blend membrane, d) FT-IR spectrum of PVA/chitosan blend membrane.

Fig. 1 compares the ATR/FT-IR spectra of GOD immobilized PVA/chitosan blend membrane (GPCB) and pure PVA/chitosan membrane(PCB). The ATR spectra for GPCB sample (b) show the same spectra for the free glucose oxidase (a) in KBr pellet. Particularly, the characteristic band due to the NH stretching of NH_2 group(3340-3360cm^{-1}) appeared in both samples. While the FT-IR spectra of GOD immobilized membrane (c) show the same peaks as the PCB membrane(d). Note the characteristic bands due to OH stretching of hydroxyl group in PVA and chitosan(3420~3460cm^{-1}) and the crystallization sensitive band of PVA at 1140cm^{-1}. The loss of band at 1140cm^{-1} as well as at 3420~3460cm^{-1} confirms the immobilization of glucose oxidase on the surface of the PVA/chitosan membrane (see spectrum(b)).

Fig. 2 exhibits the effect of glutaraldehyde on the immobilization of glucose oxidase on the membrane surface. Immobilized ratio is the ratio of GOD adhered on the membrane surface to the initial amount of GOD in the solution. The amount of immobilized enzyme on the surface of the membrane increases with the amount of glutaraldehyde. Glu-

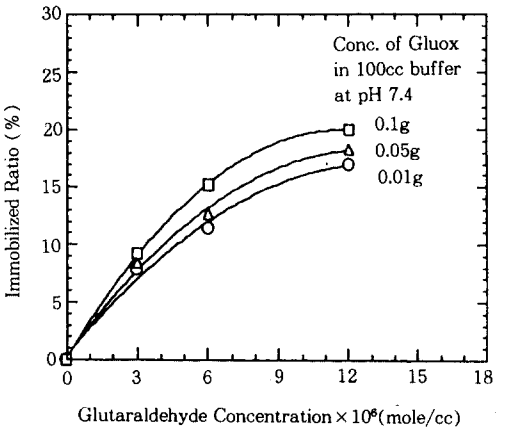


Fig. 2. Effect of the amount of glutaraldehyde on the amount of glucose oxidase immobilized the PVA/chitosan blend membrane.

taraldehyde will form a Schiff base with amino groups in chitosan and lysyl residues of the glucose oxidase. Schiff base reaction could be the primary cause of the enzyme immobilization on the PVA/chitosan membrane.

3. 2. Effect of pH and Glucose Concentration

In our previous paper[14], we reported the preparation of PVA/chitiosan blend membrane and studied the mechanical and thermal properties and swelling characteristics of the membrane. The important fact drawn from the previous studies[8,14] was that the degree of swelling changed markedly with pH ranging from 6 to 7 and was also affected by the amount of crosslinking agent applied.

Swelling measurements were first performed on the membrane prepared. Fig. 3 shows that the water content in the membrane increases with the glucose concentration in buffer solution. The membranes were prepared with an excess of enzyme so that the modest loss in activity did not affect the performance of these membranes. Note that the higher glucose concentration and thus a lower pH led to a more swollen membrane. A decrease in pH is attributed to the enzymatic reaction between GOD and glucose to form gluconic acid. In this study, we intentionally loaded much enzyme to obtain high sensitivity for glucosec concnetration in the solution. Generally the sensitivity of glucose concentration is high in a low glucose concentration (0mg%~200mg%) and is low in a high glucose concentration (above 200mg%). The leveling-off of water content in response to glucose concentration has also been observed in the development of glucose sensitive membrane which utilizes glucose oxidase. This results from the depletion of oxygen in the membrane. The oxygen limitaion is due to the fact that GOD is a flavoprotein which requires an electron acceptor such as oygen to reoxidise the flavin adenine dinucleotide that is reduced as glucose is consumed. Therefore the enzyme reaction is an oxygen-limited one at high glucose concentration. As we can see in Fig. 3, there is a little change in the pH and water content as glucose concentration is increased to 200mg% because severe O_2 depletion occurs as glucose concentration is increased.

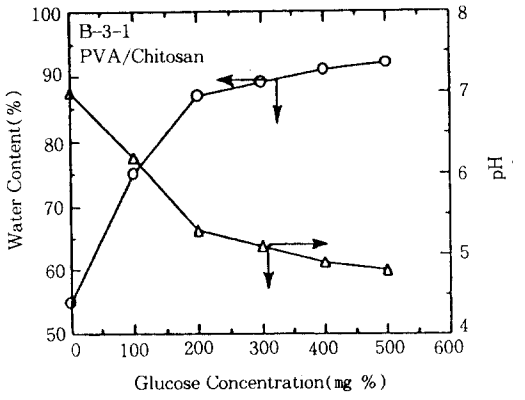


Fig. 3. Change of pH(△) and water content (○) in B-3-1 membrane in reaction between glucose and glucose oxidase.

3. 3. Insulin Permeation Through Blend Membrane

Fig. 4 illustrated results of the permeation of insulin through the GOD-immobilized blend membrane(GPCB) at a different pH buffer solution. It could be seen that the permeability of insulin through this membrane was changed with pH. The permeation rate of insulin in pH 4 buffer solution

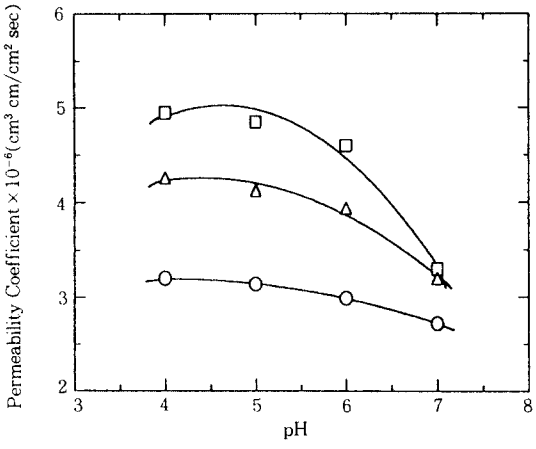


Fig. 4. Effect of pH on the permeation of insulin through PVA/chitosan blend membrane B-1-1(○), B-2-1(△), B-3-1(□).

was greater than that in pH 7. The permeability data also indicated that B-3-1 membrane, having higher water content and lower crosslinking degree, exhibited the faster permeation of insulin than that B-1-1 membrane did. The permeability decreased monotonously with increasing the concentration of crosslinking agents.

Fig. 5 showed insulin permeation through the blend membrane at a different glucose concentration. The change in permeability induced by a change in glucose concentration is very similar to a change induced by pH. This trend indicates that the production of gluconic acid catalyzed by the glucose oxidase in glucose solution might alter the pH of the solution, cause the swelling in blend membrane and consequently change the permeability. The equal and parallel change in insulin permeability caused by either pH or glucose is a significant observation because it means that a maximum change in the permeability of membrane, which can occur as a result of drop in pH in the external environment, can also be induced by the change in glucose concentration in a physiological range.

According to Horbert et al.'s relationship between the diameter of a hydrogel and permeability, the rate of 4mg insulin transport per a day is required to obtain a normal blood glucose concentration[15]. This rate is sufficient to meet the normal daily requirement of insulin for a non-insulin resistant diabetic. They also proposed that an effective implantable device could be obtained if the permeability was in the order of about 10^{-7} cm^3cm/cm^2sec for a membrane having thickness of 0.02mm ~1mm and a diameter of a hydrogel of 0.5cm~45. 3cm. In our case thickness of the present membrane was 0.1mm and diameter was 1.565cm. Therefore, our data on the blend membrane indicated that it met the requirement for such a device on the sole ground that the insulin permeability was in the order of $10^{-7}cm^3cm/cm^2sec$.

Fig. 6 shows the relationship between the logarithm of permeability and the reciprocal of the water content($1/H$) in the blend membrane. From this result and according to the theory proposed by Yasuda et al.[16], it is considered that insulin permeate through the region of free water in the swollen membrane. They also showed that a linear rela

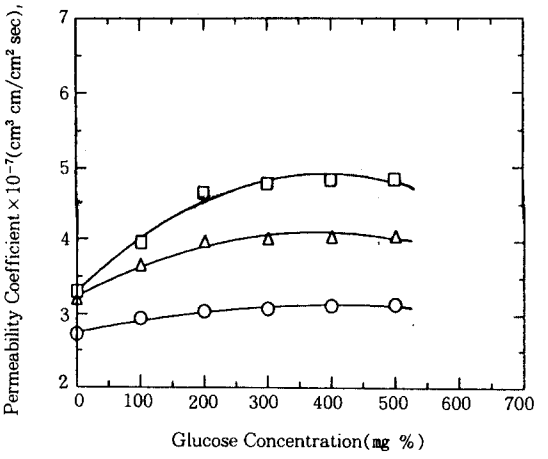


Fig. 5. Release profile of insulin through PVA/chitosan blend membranes at different glucose concentration B-1-1(○), B-2-1(△), B-3-1 (□).

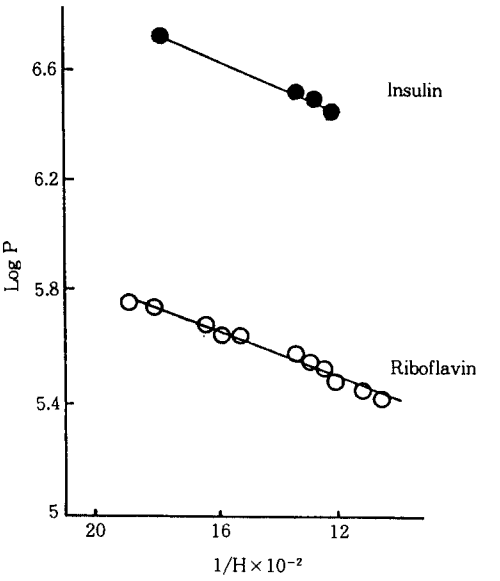


Fig. 6. Relationship between nolog P and $1/H$ in PVA/chitosan membrane system for riboflavin(○) and insulin(●).

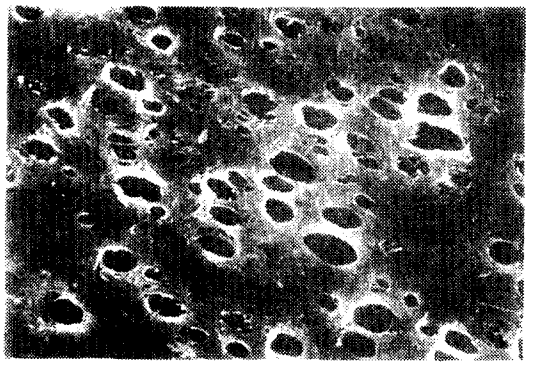
tionship between log P and $1/H$ indicated that solute might permeate through the water region contained in hydrophilic polymer membrane where no interaction between the ploymer chains took place. On this basis, this result leads to a conclusion that the permeability of insulin through the blend membrane can be controlled by a change in the water concent of the membrane caused by the degree of crosslinking.

3. 4. Insulin Permeation Through Composite Membrane

Data from Fig. 5 confirm that if glucose concentration in the solution is too high, the PVA/chitosan membrane became unsensitive to a further increase in glucose concentration, due to the oxyge depletion in the membrane. It is possible to avoid this situation by means of using an additional porous membrane placing outside the enzyme immobilized membrane. This membrane acts as a barrier to glucose permeation and therefore reduces the depletion of oxygen, because of the remarkable difference in size between the glucose and oxygen.

The SEM photograph of this membrane is shown in Fig. 7. This membrane is porous symmetric membrance having 0.2μm in pore size. Fig. 8 shows the permeability of insulin through porous membrane. The permeability of insulin through porous membrane alone was not changed by the changed in pH and was about 100 times greater than that through the PVA/chitosan membrane.

Fig. 9 illustrated the results of the permeability of insulin through composite membranes made of PVA/chitosan and nylon porous membrane in different glucose concentration. It can be seen that permeability of insulin through this membrane was changed with glucose concentration. Especially the increase of permeability is progressively responding to glucose concentration at least up to 500mg%. This explains that the more abundunt oxygen supply would allow a higher glucose concentration at a given enzyme concentration. That is, higher oxygen concentration in the membrane would be requied in order to avoid unsensitivity in higher glucose concentration.



(a) Surface of Polyamide membrane

(b) Section view of Polyamide membrane

Fig. 7. SEM view of the (a)surface, (b)cross-section of porous polyamide membrane.

4. Conclusion

Glucose oxidase immobilized PVA/chitosan membrane was prepared and the permeation of insulin through this membrane was conducted. The glucose oxidase was immobilied on the surface of the membrane. The immobilization of GOD on the membrane overcomes the problems of diffusion limitation of glucose. The water content in the membrane

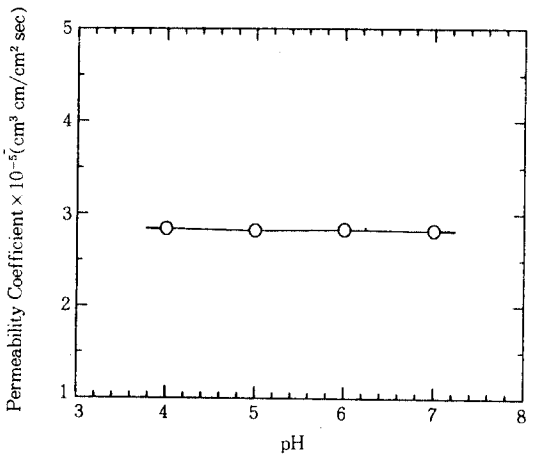


Fig. 8. Effect of pH on the permeation of insulin through porous polyamide membrane(pore size: 0.2μm).

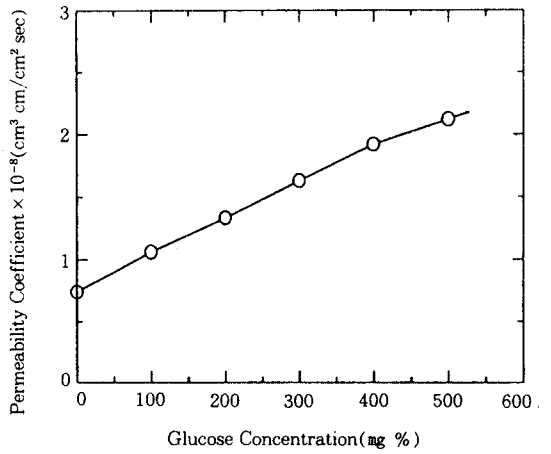


Fig. 9. Release profile of insulin through glucose oxidase immobilized composite PVA/chitosan blend membrane(B-3-1) at different glucose concentration.

showed a pH dependence. As the glucose concentration in the phosphate buffer solution increased. The pH in the solution decreased and water content in the membrane increases. The results on the permeation of insulin through GOD-immobilized PVA/chitosan blend membrane alone indicated that the permeation coefficient was in the order of 10^{-6}~10^{-7}cm^{3}cm/cm^{2}sec at pH range from 7 to 4 and were dependent on both pH and glucose concentration. In case that glucose concentration in the solution is above 200mg%, the change of water content and permeation of insulin through GOD-immobilized PVA/chitosan membrabe were negligible. Resulting from the oxygen depletion in the membrane. We avoid this problem by means of using a porous polyamide membrane. This membrane acts as a barrier to glucose permeation and therefore reduces the depletion of oxygen to glucose. The permeation of insulin through composite membrane made of PVA/chitosan and porous polyamide membrane was changed with glucose concentration in the solution. Especially the increase of permeation is in response to glucose concentration at least up to 500mg%. These results led to a conclusion that PVA/chitosan blend membrane which uses porous polyamide membrane as a glucose barrier progressively responded to glucose in the physiological range. Further research on insulin delivery through PVA/chitosan blend membrane will focus on the design and methods to achieve optimum response time.

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