Immobilization of Glucose Oxidase on Nonwoven Fabrics with *Bombyx mori* Silk Fibroin Gel

TETSUO ASAKURA,[1]* MOTOHIRO KITAGUCHI,[1] MAKOTO DEMURA,[1]
HARUTOSHI SAKAI,[2] and KEIICHI KOMATSU[3]

[1]Department of Biotechnology, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184, [2]Japan Viline Co. Ltd., Souwacho, Sashimagun, Ibaraki 306-02, and [3]Sericultural Science Research Institute, Shinjuku-ku, Tokyo 109.

SYNOPSIS

The enzyme glucose oxidase (GOD) was immobilized on the nonwoven fabrics, which have excellent properties in diffusivity of substrates, mechanical strength, and handling, with *Bombyx mori* silk fibroin gel. The nonwoven fabrics of silk fibroin, viscose rayon, polyethyleneterephthalate, 6-nylon, and polypropylene with activated surface by fluoline treatment were used. The stabilities of GOD to heat or pH changes were much improved by the immobilization as well as the case of the GOD immobilized in the silk fibroin membrane. Among nonwoven fabrics, silk fibroin was the most excellent support material for the immobilization of GOD although all nonwoven fabrics used here are able to be used as the support materials. The increase of the sensitivity was observed when the glucose sensor was made with the GOD immobilized on nonwoven silk fabrics as four times compared with the case of the GOD immobilized in the silk fibroin membrane.

INTRODUCTION

Recently, silk fibroin from *Bombyx mori* has been found to be much better than other biomaterials for the application of various new techniques such as enzyme immobilization.[1-16] As the support for such enzyme immobilization, silk fibroin has inherent advantages. In the preparation of the enzyme-immobilized membrane with silk fibroin, simultaneous insolubilization of the water-soluble membrane and immobilization of the enzyme in the membrane without any chemical reagents for crosslinking is possible.[3-9] This is based on the conformational transition of the silk fibroin chain induced easily by the treatments of drawing, compression, and immersion in alcohol or hydration under high humidity. In addition, silk fibroin can be used in various forms, such as gel, powder, fiber, and membrane, depending on the application.[1-3,6-10]

On the other hand, nonwoven fabrics have been widely used in several fields. The merit of the use of nonwoven fabrics as the support material is increase of the surface area compared with the membrane, and thus, the permeability of the substrates or products after the enzyme reaction may become large. In addition, nonwoven fabrics are easy to handle and strong mechanically. These advantages are suitable for the support of the enzyme immobilization in biosensor or bioreactor systems. Thus, it is expected that the enzyme immobilization, which maintains the excellent ability of the silk fibroin, can be attained if we can perform the enzyme immobilization with silk fibroin gel on the surface of the nonwoven fabrics.

In this study nonwoven fabrics, silk fibroin, viscose rayon, polyethyleneterephthalate, 6-nylon, and polypropylene with activated surface by fluorine treatment are used as an enzyme support material. The enzyme GOD (glucose oxidase) is immobilized on the surface of the fabrics with silk fibroin gel and the activity of the immobilized GOD is examined carefully. Finally, the enzyme-coated nonwoven fabric is applied to a glucose sensor.

* To whom correspondence should be addressed.

Journal of Applied Polymer Science, Vol. 46, 49-53 (1992)
 CCC 0021-8995/92/010049-05$04.00

EXPERIMENTAL

Materials

Several kinds of nonwoven fabrics, *Bombyx mori* silk fibroin (SF), viscose rayon (VR), polyethyleneterephthalate (PET), 6-nylon (6-N), and polypropylene (PP) with activated surface by fluorine treatment were obtained from Japan Viline Co. Ltd. The sizes and the apparent densities of all samples were 1.5 denier and 0.2 g/cm^3, respectively. The fabrics were treated with methanol by Soxhlet extractor for 12 h before use. GOD derived from *Aspergillus niger* was purchased from Wako Pure Reagents Co.

Immobilization of GOD

The aqueous solution of the silk fibroin from *B. mori* was obtained as described previously in detail.[8] Various nonwoven fabrics were soaked in the aqueous solution of silk fibroin containing the enzyme GOD and were wrung out by the apparatus for wringing. The fabrics were then allowed to stand under 50% relative humidity for 24 h. The insolubilization of the fibroin coated was performed by immersing it in 80% methanol aqueous solution for 30 s. The GOD-immobilized fabrics were stored in 0.1*M* potassium phosphate buffer of pH 7.0 at 4°C.

Water Sorption of the Fabrics

Water sorption of the nonwoven fabrics coated with the mixture, silk fibroin, and GOD on the fabrics were calculated as follow:

$$\text{Water sorption (\%)} = \frac{\text{swollen sample (g)} - \text{dry sample (g)}}{\text{dry sample (g)}} \times 100$$

where the weight of the swollen samples was measured after storage of the fabrics at 90% relative humidity for 5 days. The weights of dry sample were measured after drying under vacuum.

Measurement of the Enzyme Activity

The activity of free and immobilized GOD was assayed with D-glucose as a substrate. The change of the amount of oxygen decreased after the enzyme reaction was monitored by a Clark-type oxygen electrode (Type BO, ABLE Co. Ltd, Japan) as described previously.[8] The activity of the immobilized GOD was measured at various pH (5–8) adjusted with 0.1*M* potassium phosphate buffer to examine the pH dependence of the enzyme. The thermal stability of the immobilized GOD was examined from the enzyme activity determination performed at pH 7.0 and 25°C after heat treatment of the immobilized GOD in the phosphate buffer (pH 7) for 20 min.

Glucose Sensor

The glucose sensor was made by the combination of the GOD-immobilized nonwoven fabrics and an oxygen electrode.[8,9] The GOD-immobilized nonwoven fabric (1 × 1 cm^2) was attached on the detecting position of the oxygen electrode with an O-ring. Changes of output of the electrode after the injection of the glucose solution were recorded at 25°C. For the detection, flow system with the flow rate, 1.6 mL/min was adapted.[10]

RESULTS AND DISCUSSION

Immobilization of the Enzyme on the Nonwoven Silk Fabrics by Coating

Immobilization of GOD with silk fibroin was performed by coating a mixture of GOD and silk fibroin on the nonwoven fabrics. First, the reproducibility about the amount of the silk fibroin gel coated on the fabrics is examined by judging from the increase of the weight of the silk fabrics after a series of the preparation process. The weight increase by the coating is 6.1 within ±0.1 mg/cm^2 over 5 time trials when the SF fabric is soaked in the silk fibroin solution (2.8 w/v%). Thus, the reproducibility is very well.

Second, the increase of the weight of the silk fabrics after coating is determined by changing the initial concentration of silk fibroin from 0.8 to 3.6 w/v% (Table I). The amount of the coated silk fibroin

Table I Coating Condition of GOD with Silk Fibroin on the Nonwoven Silk Fabrics

Concentration of Silk Fibroin (w/v%)	Weight Increase[a] (mg/cm^2)
3.6	6.4
2.8	6.1
1.3	6.1
0.8	5.4

[a] Weight increase represents amount of mixture of GOD and silk fibroin adsorped on the nonwoven silk fabric. Apparent density of nonwoven silk = 0.2 g/cm^3; concentration of GOD = 2.0 w/w% of dry silk fibroin; and number of coating treatment = 1.

Table II Effect of GOD Concentration on Apparent Michaelis Constant (K_m^{app}), Apparent Maximum Activity (V_m^{app}), and Activity Yield

GOD[a] (%)	K_m^{app} (mM)	V_m^{app} (U/mg)	Activity Yield[b] (%)
2.0	8.4	6.2	5.1
0.2	7.3	29.3	24.4
0.02	6.8	60.8	50.6
0.002	5.8	113.8	94.2
free	25.2	120.0	100

[a] The amount of immobilized GOD (mg) was estimated on the basis of weight of silk fibroin coated on the fabric and mixing ratio of GOD and silk fibroin (2 w/v%).

[b] Activity yield represents the immobilized GOD activity relative to that of the free enzyme.

increases gradually with increasing the initial concentration of silk fibroin. In addition, the leakage of the silk fibroin gel was not detected by the Lowry method[17] within experimental error after one month.

Activity of the Immobilized GOD on Nonwoven Silk Fabrics

The apparent Michaelis constant, K_m^{app}, apparent maximum activity, V_m^{app}, and the activity yield of the immobilized GOD on the nonwoven silk fabric were listed as a function of the concentration of GOD in the silk fibroin solution before immersing the nonwoven fabrics (Table II). The K_m^{app} values of the immobilized GOD are considerably smaller than the corresponding value of free GOD and tend to decrease with decreasing the GOD concentration. The V_m^{app} values increase with decreasing the GOD concentration and reach the value of free GOD at 0.002% GOD. The activity yield of the GOD immobilized on nonwoven silk fabric also increases with decreasing the GOD concentration. In particular, the activity yield of the GOD immobilized was more than 90% when 0.002% GOD was coated on the fiber with silk fibroin gel. These indicate that the diffusion of the substrate glucose may be a rate-determining step in the enzyme reaction as well as the silk fibroin membrane.[2] Moreover, the relative activity of GOD immobilized on the silk fiber surface becomes higher than that immobilized in the membrane when the concentration of GOD is same.[18] This superiority of the use of the nonwoven silk as a support comes from the increase of the surface area. The thermal and pH stabilities of the free and immobilized GOD on the nonwoven silk fabric were much improved as well as that in the silk fibroin membrane reported previously.[8] In addition, the initial activity of the immobilized GOD was kept for one month.

Immobilization of GOD on Various Nonwoven Fabrics by Coating

The K_m^{app}, V_m^{app}, and relative activities of the enzymes were determined as well as the water sorption as listed in Table III. The water sorption increases in the order of VR > SF > 6-N > PET > PP. In general, relatively hydrophilic fiber has high water sorption ability as is expected. The values of K_m^{app}, V_m^{app}, and the relative activity of the immobilized GOD are high for SF and VR, which are correlated to the high water sorption.

Figure 1 shows the thermal stabilities of free and

Table III Water Sorption, Apparent Michaelis Constant (K_m^{app}), Apparent Maximum Activities (V_m^{app}),[a] and Relative Activity of the Immobilized GOD on Various Nonwoven Fabrics[b]

Fabrics	Water Sorption of Fabrics (%)[c]	K_m^{app} (mM)	V_m^{app} (U/mg)	Relative Activity (%)
SF	23.3	8.4	11.4	100
VR	30.9	8.2	10.7	94
6-N	10.2	5.8	8.2	72
PET	8.3	5.6	8.3	73
PP	1.0	4.5	8.6	75

[a] The amount of immobilized GOD (mg) was estimated on the basis of the weight of silk fibroin coated on each fabric containing 2% GOD.

[b] Apparent density of nonwoven fabrics used was 0.2 g/cm^3.

[c] Water sorption was equilibrium value after storage at 90% of relative humidity and 25°C.

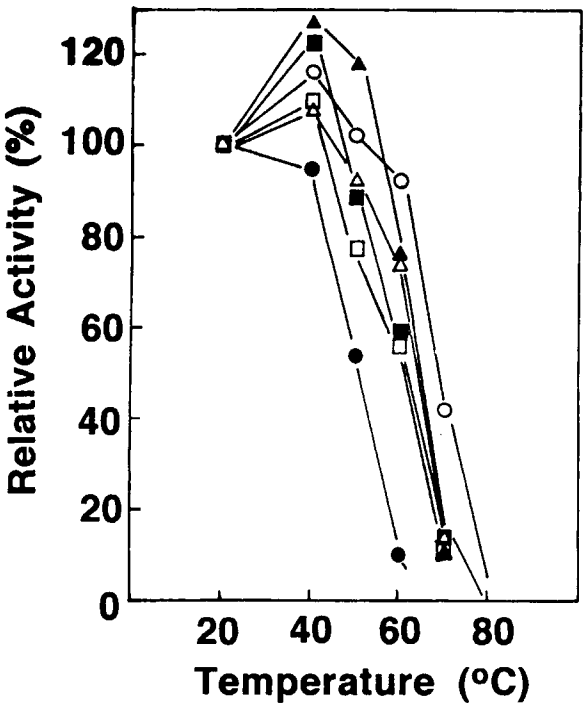

Figure 1 Thermal stabilities of free and immobilized GOD on various nonwoven fabrics: ● free, ○ SF, ■ VR, ▲ 6-N, △ PET, □ PP.

immobilized GOD on various fibers. The thermal stability of the GOD was much improved by immobilization in all cases. In particular, the thermal stability of GOD immobilized on nonwoven silk is the best among them.

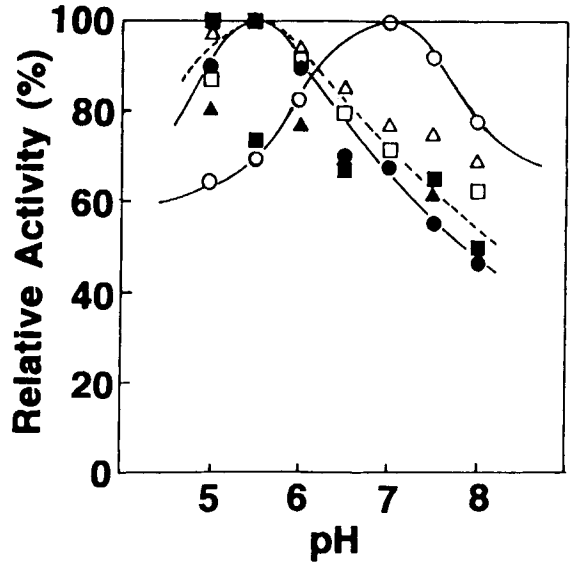

Figure 2 pH dependences of the relative activities of free and immobilized GOD on the nonwoven fabrics: ● free, ○ SF, ■ VR, ▲ 6-N, △ PET, □ PP.

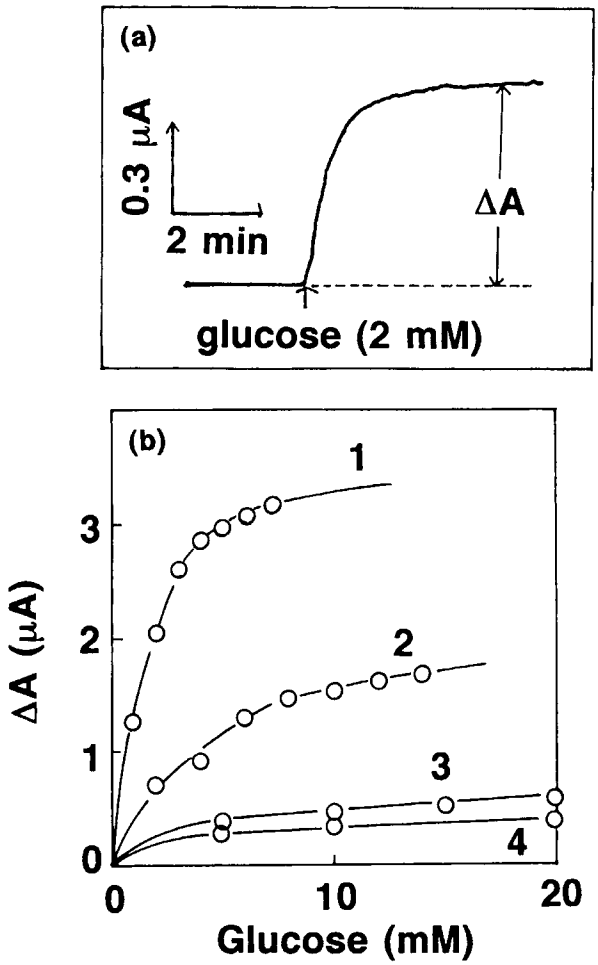

Figure 3 Typical response of (a) the glucose sensor with the GOD-immobilized nonwoven SF and (b) the outputs at the steady-state (ΔA) as a function of glucose concentration. 1, 2, 3, and 4 represent the concentration of GOD to the weight of silk fibroin gel coated at 2, 0.2, 0.02, and 0.002%, respectively.

The pH dependences of the relative activities of free and immobilized GOD are shown in Figure 2. The optimum pH value of the immobilized GOD does not change within experimental error compared with the value, pH = 5.5, for free GOD except for the case of nonwoven silk fabrics. For the silk fabrics the optimum pH shift is to the relatively basic side (pH = 7.0) as described above. This might be due to the property of silk fibroin as an anionic protein. Thus, judging from the thermal and pH stabilities of the immobilized GOD, the nonwoven SF fabric is the best material for the immobilization of GOD.

Application to Glucose Sensor

Figure 3(a) shows the typical response of the glucose sensor with the GOD-immobilized nonwoven SF.

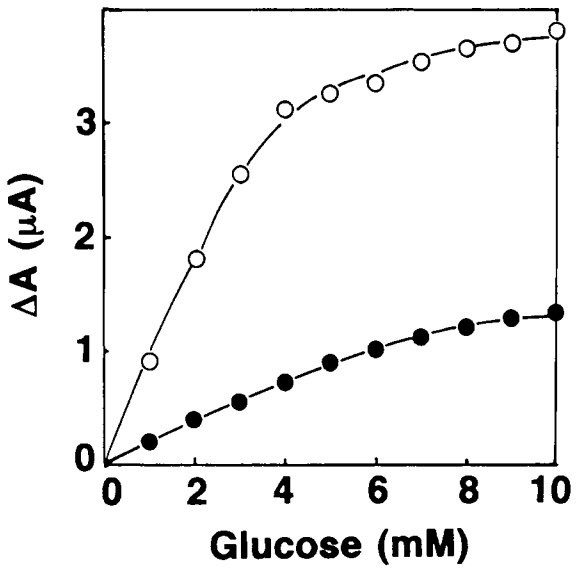

Figure 4 A comparison of outputs of glucose sensors with the nonwoven SF and the silk fibroin membrane. The amount of the used GOD in the silk fibroin gel and the membrane were same (6×10^{-3} mg). ○ nonwoven fabric, ● membrane.

When the glucose solution (2 mM) was added in the cuvette at the upward arrow, the sensor responded rapidly as decrease in oxygen pressure. The outputs at the steady state (ΔA) were plotted against glucose concentration in the cuvette [Fig. 3(b)]. With increasing the concentration of GOD in the silk fibroin gel, the sensitivity of this sensor becomes higher. The response of glucose sensor with other nonwoven fabrics made from artificial fibers is essentially the same of that with the SF.

The outputs of glucose sensors with the nonwoven SF and the silk fibroin membrane were compared (Fig. 4). In this case the amounts of the immobilized GOD on the fabrics was quantitatively determined using spin labeling ESR method.[18] It is found that the output for nonwoven silk is about four times that of the membrane when the concentration of substrate is the same between them. This is due to the increase of the surface area of the support, immobilization of the GOD at the surface, and high diffusivity of substrate in the nonwoven matrix compared with the silk fibroin membrane. Thus, the enzyme immobilization on the surface of nonwoven fiber with silk fibroin gel is very useful in developing the biosensor system. The mechanical strength of the nonwoven fiber is sufficiently high.

In conclusion, silk fibroin gel and various nonwoven fabrics were used as an enzyme support material. Judging from the thermal and pH stabilities of the immobilized GOD, the nonwoven silk fibroin (SF) fabrics is the best material for the immobilization of GOD. The GOD-immobilized SF nonwoven fabric was applied to a glucose sensor. The output for the nonwoven SF is about four times of that of the membrane when the concentration of substrate is same between them. Thus, the use of nonwoven fiber and silk fibroin gel is very useful in developing the enzyme immobilization system.

This work was partially supported by Tokuyama Science Foundation, Japan.

REFERENCES

1. T. Asakura, M. Demura, H. Ogawa, K. Matsushita, and M. Imanari, *Macromolecules,* **24,** 620 (1991).
2. M. Demura and T. Asakura, *J. Membrane Sci.,* **59,** 39 (1991).
3. M. Demura, T. Kumura, and T. Asakura, *Bioelectrochem. Bioenerg.,* **26,** 167 (1991).
4. T. Asakura, H. Yoshimizu, and M. Kakizaki, *Biotechnol. Bioeng.,* **35,** 511 (1990).
5. M. Demura, T. Komura, T. Hiraide, and T. Asakura, *Sen-i Gakkaishi,* **46,** 391 (1990).
6. H. Yoshimizu and T. Asakura, *J. Appl. Polym. Sci.,* **40,** 127 (1990).
7. A. Kuzuhara, T. Asakura, R. Tomoda, and T. Matsunaga, *J. Biotech.,* **5,** 199 (1987).
8. M. Demura and T. Asakura, *Biotechnol. Bioeng.,* **33,** 598 (1989).
9. M. Demura, T. Asakura, and T. Kuroo, *Biosensors,* **4,** 361 (1989).
10. M. Demura, T. Asakura, E. Nakamura, and H. Tamura, *J. Biotech.,* **10,** 113 (1989).
11. T. Asakura, H. Yoshimizu, A. Kuzuhara, and T. Matsunaga, *J. Seric. Sci. Jpn.,* **57,** 203 (1988).
12. T. Asakura, J. Kanetake, and M. Demura, *Polym. Plast. Technol. Eng.,* **28,** 453 (1989).
13. L. Grasset, D. Cordier, and A. Ville, *Process Biochem.,* **14,** 2 (1979).
14. S. Miyairi, M. Sugiura, and S. Fukui, *Agric. Biol. Chem.,* **42,** 1661 (1978).
15. L. Grasset, D. Cordier, and A. Ville, *Biotechnol. Bioeng.,* **19,** 611 (1977).
16. D. Cordier, R. Couturier, L. Grasset, and A. Ville, *Enzyme Microb. Technol.,* **4,** 249 (1982).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.,* **195,** 265 (1953).
18. T. Asakura, H. Kitaguchi, M. Demura, H. Sakai, M. Kaneko, A. Kurioka, and K. Komatsu, *J. Seric. Sci. Jpn.,* **60,** 466 (1991).

Received July 22, 1991
Accepted October 30, 1991