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COVALENT IMMOBILIZATION OF GLUCOSE OXIDASE ON MAGNETITE PARTICLES VIA GRAFT POLYMERIZATION OF ACRYLIC ACID

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ABSTRACT

A new technique for immobilizing enzyme molecules on magnetite particles via the graft polymerization of acrylic acid is presented. The polymerization of acrylic acid was carried out in a redox system consisting of ceric ion and mercapto groups introduced onto magnetite particles. In the course of the polymerization, poly(acrylic acid) was attached to the magnetite particles. Glucose oxidase was covalently immobilized on the magnetite particles by the condensation reaction with the carboxyl groups of the poly(acrylic acid). It was shown that 2.8 mg of glucose oxidase was immobilized on 1 g of the magnetite attached with poly(acrylic acid), and the immobilized glucose oxidase had a specific activity of 81 units/mg, which was 50% of that of the native enzyme. Due to the immobilization, the optimum pH for glucose oxidase was shifted to a higher value and the temperature dependency of activity was diminished. A kinetic study of the glucose oxidation reaction with immobilized enzyme showed that the immobilization limited accessibility of glucose molecules to the active sites of the enzyme and caused a decrease of the maximum reaction rate. Glucose oxidase immobilized on magnetite particles kept 95% of its original activity in water over a period of 9 months.

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INTRODUCTION

Enzymes have been fitted on a variety of supports by immobilization for many practical purposes [1–7]. Many kinds of insoluble substances, including inorganic substances [8–10], have been used as support materials. Magnetism, one of the important properties of these support materials, provides a new enzyme-handling technique in the field of reactor applications. If an enzyme is immobilized on particles of magnetizable materials, such as magnetite, transport of the enzyme into a reactor or recovery from a reaction mixture can be carried out magnetically.

Attaching polymer chains by graft polymerization for the purpose of immobilizing enzymes on such inorganic materials [11–15] can be employed as a useful method of modifying an inorganic surface. For example, polymerization of a vinyl monomer in a redox system consisting of ceric ion and reducing groups introduced onto inorganic particles has been attempted, and it has been shown that polymer chains can be attached to the particles [12, 14]. If an attached polymer has functional groups, such as carboxyl, amino, and aldehyde groups, they can be applied to the covalent immobilization of enzymes.

In this paper a new technique for immobilizing enzymes on inorganic magnetizable particles via graft polymerization is presented. Poly(acrylic acid) chains were attached to magnetite particles by the redox polymerization of acrylic acid, and covalent enzyme immobilization on the magnetite particles was demonstrated by the condensation reaction of glucose oxidase (GOD) with the carboxyl groups of the polymer chains attached to the particles. The activity of the immobilized GOD was measured under various conditions and compared with that of native GOD.

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EXPERIMENTAL

Materials

The magnetite used in this work was MAT-305 obtained from Toda Kogyo Corp. It was in the form of spherical particles. The magnetite had an average particle size of 0.23 μm and a BET surface area of 7.2 m^2/g. The GOD used (EC 1.1.3.4, grade II, from *Aspergillus* sp.) was supplied by Toyobo Co.; it had an activity of 164 units/mg. The peroxidase (POD) used (EC 1.11.1.7, type I, from horseradish) was supplied by Sigma Chemical Co.; it had an activity of 120 units/mg. Acrylic acid was purchased from Wako Pure Chemical Ind., and purified by distillation under reduced pressure prior to use. 3-Mercaptopropyltrimethoxysilane (MPS) from Kanto Chemical Co., was used without further purification. Other chemicals were guaranteed reagent grade or analytical grade commercial materials and used without further purification.

Attaching Poly(Acrylic Acid) to Magnetite Particles

The attachment of poly(acrylic acid) was carried out by following the scheme shown in Fig. 1. The magnetite particles were treated with MPS to introduce mercapto groups onto their surfaces as described in a previous publication [12]. Attaching poly(acrylic acid) by redox polymerization was conducted as follows: Into a flask, 0.5 g of the magnetite treated with MPS, 3.0 g of acrylic acid, and 10.0 mL of

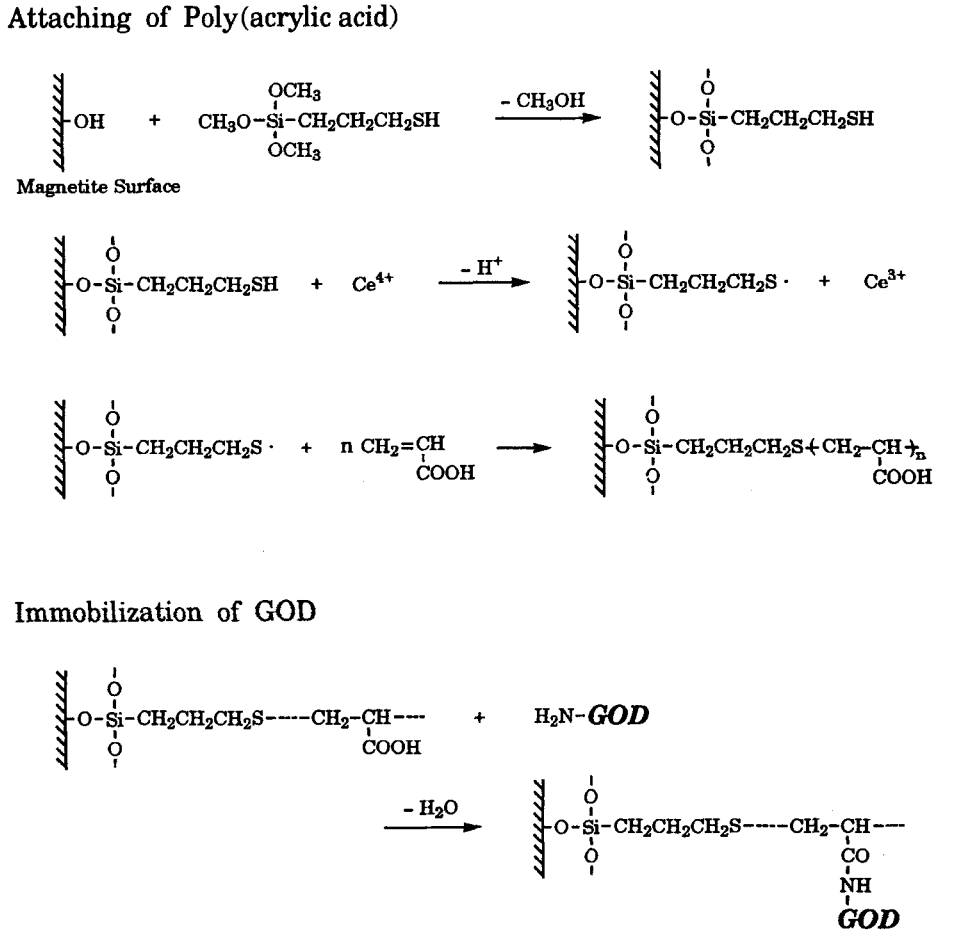

FIG. 1. Attaching of poly(acrylic acid) to the surface of magnetite and immobilization of GOD.

distilled water were charged. After deaeration of the mixture, a solution of 0.2 mmol of ceric ammonium nitrate in 3.0 mL of 1 N nitric acid was added. The polymerization was carried out at 30°C with stirring under nitrogen. After a given time the polymerization was stopped by addition of hydroquinone. The reaction mixture was diluted with distilled water and centrifuged at 10^5 m/s^2 until the magnetite particles were completely precipitated. The precipitated magnetite particles were dispersed in distilled water and centrifuged once more. This procedure was repeated several times, and the precipitated particles were dried at a temperature below 60°C in vacuo.

The amount of attached poly(acrylic acid) was determined from the weight increase of the magnetite. The conversion of acrylic acid into the polymer was determined from the sum of the amount of attached polymer and the amount of unattached polymer.

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Immobilization of GOD on Magnetite Particles

GOD was immobilized on magnetite particles attached to poly(acrylic acid) by the condensation reaction with carboxyl groups of the polymer as illustrated in Fig. 1. 1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (CMC) was used as a condensing agent according to the method of Mosbach [2].

A mixture of 0.5 g of the magnetite attached to poly(acrylic acid), 17 mg of GOD, and 7.0 mL of 0.1 M phosphate buffer (pH 6.5) was placed into a flask and stirred for 5 minutes. Subsequently, 80 mg of CMC was added and stirring was continued. After 18 hours of stirring, the reaction mixture was centrifuged at 10^5 m/s^2, and the magnetite particles were completely precipitated. The precipitated particles, i.e., GOD-bound magnetite, were dispersed in distilled water, filtered off, and washed on a filter with distilled water. This washing procedure was repeated several times. The GOD-immobilizing reaction and the succeeding washing were conducted at 4°C.

The GOD-bound magnetite thus prepared, as well as the raw magnetite and the magnetite attached to poly(acrylic acid), was analyzed by means of IR spectroscopy. IR spectra were recorded on a Shimadzu FTIR-8100M spectrometer.

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Determination of Immobilized GOD

The amount of immobilized GOD was estimated by analysis with Folin-Ciocalteu phenol reagent after alkaline copper treatment according to the method of Lowry [16].

Fifty milliliters of a 2.0% solution of Na_2CO_3 in a 0.1 N NaOH solution and 1.0 mL of a 0.5% solution of $CuSO_4 \cdot 5H_2O$ in a 1.0% sodium tartrate solution were mixed, and 2.5 mL of the mixture was added to a suspension of 5.0 mg of GOD-bound magnetite particles in 0.5 mL of distilled water. The suspension was stirred and allowed to stand for 10 minutes at room temperature. Folin-Ciocalteu phenol reagent (0.25 mL) diluted to 1 N in acid was added to the suspension very rapidly and mixed within a second or two. After 30 minutes or longer, the magnetite particles in the suspension were filtered off, and the filtrate was subjected to colorimetry. Absorbance at 660 nm was measured on a Hitachi U-2000 spectrometer. The amount of immobilized GOD was calculated from a standard curve obtained with solutions of 10–250 μg of the native GOD in 0.5 mL of distilled water.

GOD Activity Measurements

The activity of immobilized GOD was measured by the colorimetric method based on the procedure of Trinder [17]. The method includes the reaction of hydrogen peroxide, produced in oxidation of glucose by GOD, with phenol and 4-aminoantipyrine in the presence of POD to yield a colored product. The amount of colored product is regarded as a measure of the activity of GOD.

In a dark bottle, 32.0 mg of 4-aminoantipyrine and 2.5 mg of POD were dissolved in 200 mL of 0.1 M phosphate buffer, and a solution of 4.2 mg of phenol in 4.0 mL of distilled water was added: Solution 1. A D-glucose solution of 1.0 mM was prepared and allowed to stand for 12 hours or more at room temperature: Solution 2. While avoiding direct sunlight, 5.0 mL of Solution 1 and 0.5 mL of

Solution 2 were kept at a given temperature and mixed with 1.0 mg of GOD-bound magnetite particles, and the mixture was incubated at the given temperature. After 60 minutes the mixture was cooled to 0°C and incubated for 5 minutes. Then the magnetite particles were precipitated rapidly with a magnet, and the supernatant was subjected to absorbance measurement at 505 nm. The activity of immobilized GOD was calculated from a standard curve obtained with 1–25 μg of the native GOD. The activity was measured over the 4–9 pH range and over the 20–60°C temperature range.

The colorimetric method, on the other hand, was applied to measurements of the GOD reaction rate at various glucose concentrations. Data were employed to discuss the kinetic effect of the immobilization.

RESULTS AND DISCUSSION

Attaching of Poly(Acrylic Acid) to Magnetite Particles by Redox Polymerization

It was determined by elemental analysis that the amount of mercapto groups introduced onto the magnetite particles was 0.12 mmol/g. Figure 2 shows the result of polymerization of acrylic acid in a redox system consisting of ceric ion and the mercapto groups introduced onto the magnetite particles; a considerable rate of the polymerization is seen. After polymerization for an hour, ca. 20% of acrylic acid was converted into poly(acrylic acid), and a portion of the polymer was attached to the magnetite particles. The amount of attached polymer was 60 mg/g of the magnetite employed in the polymerization. On the other hand, it was observed that elongation of the polymerization time caused gel formation. In the case of polymerization for 2 hours, for example, the reaction mixture gelled and lost fluidity, and the magnetite particles contained in the mixture could not be isolated. In addition, the

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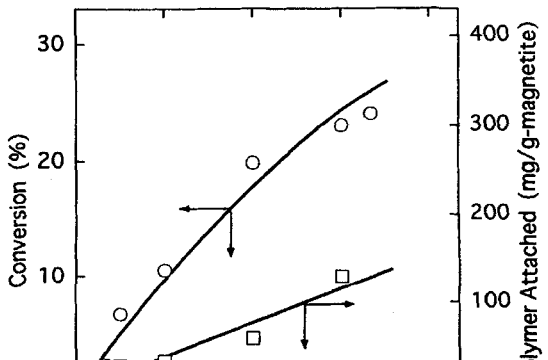

FIG. 2. Polymerization of acrylic acid in the redox system consisting of ceric ion and mercapto groups introduced onto the surface of magnetite.

polymerization of acrylic acid was not initiated in the presence of untreated magnetite particles, and no polymer was attached to the particles.

Immobilization of GOD on Magnetite Particles Attached with Poly(Acrylic Acid)

Although GOD was immobilized on the magnetite particles attached with poly(acrylic acid) by condensation reaction with carboxyl groups of the polymer, no GOD was immobilized on untreated magnetite particles or the magnetite particles only treated with MPS. The amount and the activity of GOD immobilized on the magnetite particles are given in Table 1. The immobilized GOD had a specific activity of 81 units/mg, which was 50% of the native GOD's.

In Fig. 3 the IR spectrum of the GOD-bound magnetite particles is shown together with that of the untreated magnetite particles, the magnetite particles attached with poly(acrylic acid), and the native GOD. In the IR spectrum of the magnetite attached with the polymer, an absorption in the vicinity of 1730 cm^{-1}, corresponding to C=O bond stretching of carboxyl groups, was clearly observed. In the spectrum of the GOD-bound magnetite, slight absorptions, which seem to be due to the immobilized GOD, were observed in the range from 1500 to 1600 cm^{-1}, although the spectrum was similar to that of the magnetite attached with poly(acrylic acid).

Effect of pH and Temperature on Activity of GOD Immobilized on Magnetite Particles

Figure 4 shows the effect of pH on the activity of the native and immobilized GOD. The optimum pH for the native GOD was between 5.0 and 7.0, in accord with the result described elsewhere [18]. However, the optimum pH for the immobilized GOD was in the vicinity of 7.0. This shift of optimum pH by immobilization could be attributed to the influence of the remaining carboxyl groups of poly(acrylic acid) attached to the magnetite particles. Assuming that at most a few amino groups per GOD molecule reacted with carboxyl groups of the polymer attached to the magnetite, it can be seen from the data given in Table 1 that less than 1% of total

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TABLE 1. Amount and Activity (at 30°C, pH 7.0) of Immobilized GOD

Support	GOD immobilized, mg/g-support	Apparent activity, units/g-product	Specific activity, units/mg-GOD immobilized
Untreated Fe_3O_4	0	0	–
Fe_3O_4-SH[a]	0	0	–
Fe_3O_4-pAA[b]	2.8	226	81

[a]Mercapto groups were introduced by treatment with MPS.

[b]The polymerization of acrylic acid was carried out for 60 minutes in the presence of magnetite particles treated with MPS (Fe_3O_4-SH). The amount of poly(acrylic acid) attached to the magnetite was 60.0 mg/g-magnetite.

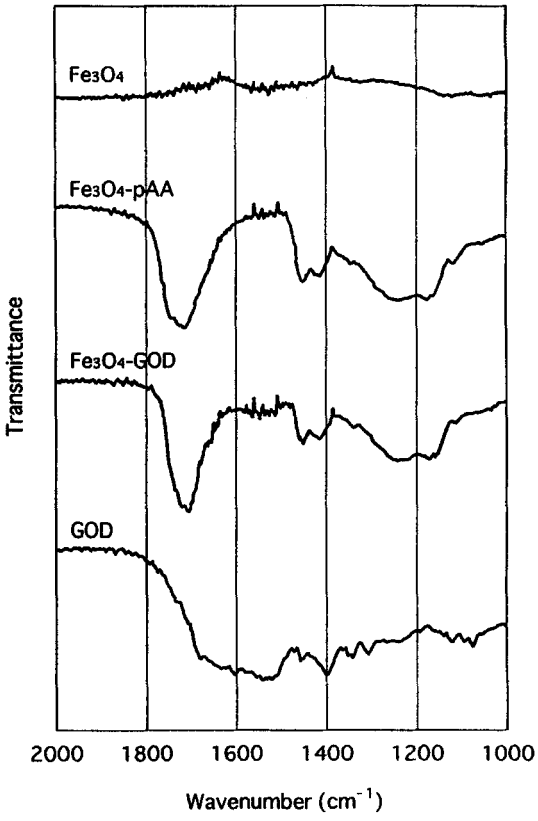

FIG. 3. IR spectra: Fe_3O_4, untreated magnetite; Fe_3O_4-pAA, magnetite attached with poly(acrylic acid); Fe_3O_4-GOD, GOD-bound magnetite; GOD, native GOD.

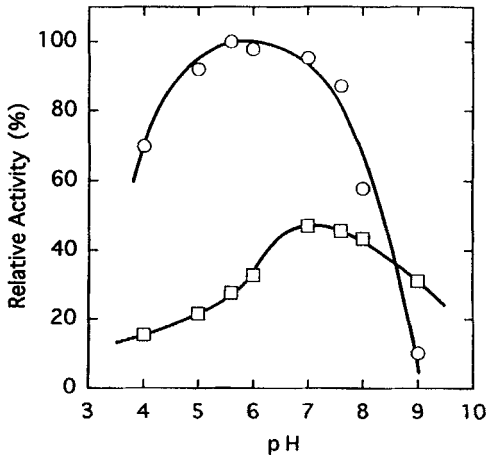

FIG. 4. Effect of pH on the activity of GOD: (○) native, (□) immobilized on magnetite.

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carboxyl groups of the polymer was consumed in the condensation reaction with GOD, and that the remaining carboxyl groups were still free. Therefore, the shift of optimum pH can be regarded as equivalent to canceling the acidity due to the remaining carboxyl groups.

The effect of temperature on the activity of the native and immobilized GOD is shown in Fig. 5. While the activity of the native GOD considerably depended on temperature and the optimum temperature was between 30 and 40°C, the activity of the immobilized GOD did not show such a remarkable temperature dependency. It is an interesting result that the activity of the immobilized GOD was almost constant over the 30 to 60°C temperature range, and higher than that of the native one at 60°C. Although the question of why the temperature dependency of GOD activity is influenced by immobilization is still under investigation, a possible explanation of the difference between the temperature-activity profiles of the native and immobilized GOD may involve conformational changes of GOD molecules caused by covalent immobilization on magnetite particles.

Kinetic Effect of Immobilization

Assuming that the glucose oxidation with GOD proceeds through a Michaelis-Menten mechanism, the reaction is

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

where E, S, ES, and P represent the enzyme (GOD), the substrate (β-D-glucose), the enzyme-substrate complex, and the product (D-glucono-δ-lactone), respectively. The rate constants are given by k_1, k_2, and k_3. If the steady state where the concentration [ES] is constant, i.e., k_1[E][S] is equal to (k_2 + k_3)[ES], is assumed, the glucose oxidation rate V (= k_3[ES]) is given by

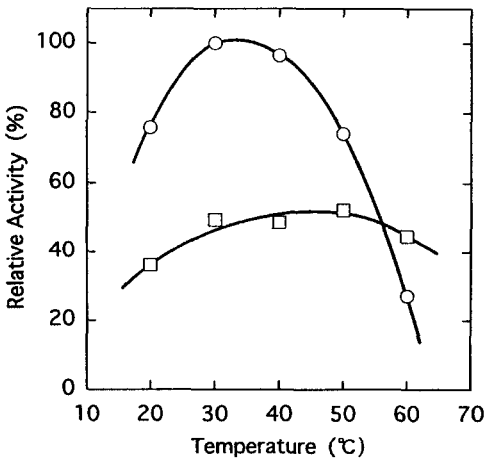

FIG. 5. Effect of temperature on the activity of GOD: (○) native, (□) immobilized on magnetite.

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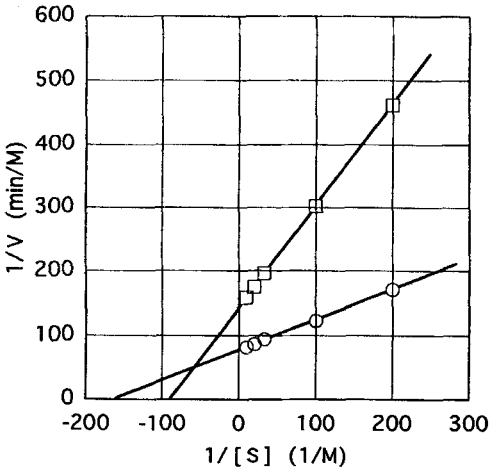

FIG. 6. Lineweaver-Burk plots for glucose oxidation (at 30°C, pH 7.0) by GOD: (○) native, (□) immobilized on magnetite. The concentration of GOD was 90 mg/L.

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$$V = V_{max}[S]/(K_m + [S])$$
$$V_{max} = k_3[E]_0$$
$$K_m = (k_2 + k_3)/k_1$$

where $[E]_0$ represents the initial concentration of the enzyme, which is equal to [E] + [ES]. V_{max} and K_m are the maximum reaction rate and the Michaelis constant, respectively. The reciprocal of the rate V is presented by

$$\frac{1}{V} = \frac{(K_m/V_{max})}{[S]} + \frac{1}{V_{max}}$$

which means that plots of $1/V$ against 1/[S] (Lineweaver-Burk plots) give a straight line, and the intercepts on the $1/V$ axis and the 1/[S] axis give the values of $1/V_{max}$ and $-1/K_m$, respectively.

In order to study the kinetic effect of immobilization, the rates of glucose oxidation reaction by the native and immobilized GOD were measured at various glucose concentrations. Figure 6 shows Lineweaver-Burk plots for glucose oxidation by the native and immobilized GOD. The plots gave straight lines, typical of the Michaelis-Menten form.

The maximum reaction rates V_{max} and the apparent Michaelis constants K_m as determined from Fig. 6 are presented in Table 2. The K_m value for the GOD immobi-

TABLE 2. Kinetic Parameters for Native and Immobilized GOD

GOD	V_{max}, mM/min	K_m, mM
Native	12.8	6.1
Immobilized	6.9	11.3

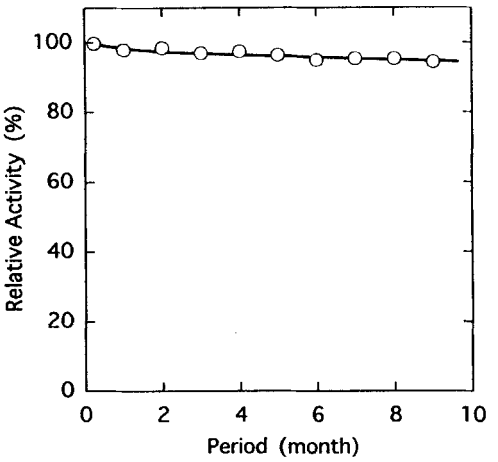

FIG. 7. Stability of GOD immobilized on magnetite.

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lized on magnetite particles was larger than that for the native GOD. The larger value of K_m for the immobilized GOD suggests a decrease of the rate constant k_1 due to limited accessibility of glucose molecules to active sites of the immobilized GOD, which may be associated with conformational changes of GOD molecules caused by the immobilization. On the other hand, the decrease of V_{max} value by immobilization is considered to be the result of a decrease of the rate constant k_3.

Stability of Immobilized GOD

The stability of the GOD immobilized on magnetite particles was examined in water (pH 7.0). A mixture of 0.5 g of the GOD-bound magnetite and 60 mL of distilled water was stored at 4°C in the dark, and the activity of the immobilized GOD was measured periodically.

Under the conditions in which the GOD-bound magnetite was stored, little decrease of the activity was observed. As shown in Fig. 7, the immobilized GOD kept 95% of its original activity in water over a period of 9 months. The result suggests that both denaturation of the immobilized GOD and isolation of the GOD from the magnetite particles did not occur markedly over the period.

CONCLUSIONS

Poly(acrylic acid) was attached to the surface of magnetite by the polymerization of acrylic acid in a redox system consisting of ceric ion and mercapto groups introduced onto the surface. The carboxyl groups of the polymer were applied to covalent immobilization of glucose oxidase on the surface. The immobilized enzyme had a specific activity of 50% of the native enzyme's and kept 95% of its original activity in water over a period of 9 months.

The immobilization of enzymes on magnetite particles enables the magnetic transport of enzymes to be carried out in the field of reactor applications. The

immobilizing technique presented in this paper, on the other hand, can be applied to modification of inorganic materials with a variety of functional proteins [19, 20]. For example, if the immobilizing technique is applied to the introduction of an antibody onto the surface of a piezoelectric crystal, detection of the antigen will be performed by the piezoelectric immunosensing system.

REFERENCES

[1] A. B. Patel, S. N. Pennington, and H. D. Brown, *Biochim. Biophys. Acta, 178*, 626 (1969).
[2] K. Mosbach, *Acta Chem. Scand., 24*, 2084 (1970).
[3] J. B. Taylor and H. E. Swaisgood, *Biochim. Biophys. Acta, 284*, 268 (1972).
[4] M. Valaris and W. J. Harper, *J. Food Sci., 38*, 477 (1973).
[5] P. Gemeiner, C. Polak, A. Breier, and L. Petrus, *Enzyme Microbiol., Technol., 8*, 109 (1986).
[6] N. F. Almeida, E. J. Beckman, and M. M. Ataai, *Biotechnol. Bioeng., 42*, 1037 (1993).
[7] S.-A. Wilson, K. Peek, and R. M. Daniel, *Ibid., 43*, 225 (1994).
[8] W. F. Line, A. Kwong, and H. H. Weetall, *Biochim. Biophys. Acta, 242*, 194 (1971).
[9] G. Baum, F. B. Ward, and H. H. Weetall, *Ibid., 268*, 411 (1972).
[10] F. Borrego, M. Tari, A. Manjon, and J. L. Iborra, *Appl. Biochem. Biotechnol., 22*, 129 (1989).
[11] R. Laible and K. Hamann, *Adv. Colloid Interface Sci., 13*, 65 (1980).
[12] N. Tsubokawa, K. Maruyama, Y. Sone, and M. Shimomura, *Polym. J., 21*, 475 (1989).
[13] N. Tsubokawa, A. Kogure, K. Maruyama, Y. Sone, and M. Shimomura, *Ibid., 22*, 827 (1990).
[14] N. Tsubokawa and K. Seno, *J. Macromol. Sci.—Pure Appl. Chem., A31*, 1135 (1994).
[15] N. Tsubokawa, Y. Shirai, H. Tsuchida, and S. Honda, *J. Polym. Sci., Part A, 32*, 2327 (1994).
[16] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem., 193*, 265 (1951).
[17] P. Trinder, *Ann. Clin. Biochem., 6*, 24 (1969).
[18] H. J. Bright and M. Appleby, *J. Biol. Chem., 244*, 3625 (1969).
[19] H. Muramatsu, J. M. Dicks, E. Tamiya, and I. Karube, *Anal. Chem., 59*, 2760 (1987).
[20] B. König and M. Grätzel, *Anal. Chim. Acta, 281*, 13 (1993).

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