Entrap-Immobilization of Invertase on Composite Gel Fiber of Cellulose Acetate and Zirconium Alkoxide by Sol–Gel Process

KOJI NAKANE,[1] TAKASHI OGIHARA,[1] NOBUO OGATA,[1] YOUICHI KUROKAWA[2]

[1] Department of Materials Science and Engineering, Faculty of Engineering, Fukui University, Bunkyo 3-9-1, Fukui 910-8507, Japan

[2] Division of Materials Chemistry, Graduate School of Engineering, Tohoku University, Aramaki, Aoba-ku, Sendai 980-8579, Japan

Received 5 June 2000; accepted 15 September 2000

ABSTRACT: We prepared a composite gel fiber by the gel formation of cellulose acetate and zirconium tetra-*n*-butoxide. Gel fiber is stable in common solvents, phosphate solution, and electrolyte solution. Invertase was entrap-immobilized on the gel fiber. The immobilization was easily performed under the mild conditions. The apparent Michaelis constant (K_m) and maximum reaction velocity (V_{max}) were estimated from Eadie–Hofstee plot for immobilized invertase. The K_m of immobilized invertase was larger than that of native invertase, while the opposite tendency was observed for the V_{max}. The activity for the immobilized invertase became higher with increasing fiber diameter. It indicates that the hydrolysis of sucrose occurs in the neighborhood of the fiber surface. The thermal stability of the immobilized invertase was higher than those of its native counterpart. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 81: 2084–2088, 2001

Key words: cellulose acetate; zirconium tetra-*n*-butoxide; fiber; invertase; entrap-immobilization

INTRODUCTION

There have been many investigations of enzyme immobilization.[1] Immobilization of invertase is one of the important subjects of this research.[2–9] In glutamic acid fermentation, with sugarcane molasses as the main carbon source, the hydrolysis of the molasses to glucose and fructose with invertase is effective for a higher glutamic acid production yield. In addition, a hydrolyzed sugar mixture obtained by invertase has the advantage of being colorless as compared to the colored products obtained by acid hydrolysis. To develop a practical immobilized invertase system for this process, Yoshii et al. examined the polymer matrices (polyurethane, photosetting resin, κ-carrageenan, vegetable gelatin, and Na–alginate) for the immobilization of invertase.[9] It is recognized that Na–alginate is the best matrix for invertase immobilization because of its low activity loss during immobilization and enzyme reaction.

Alginate and κ-carrageenan gels are widely used as an entrapping matrix for enzyme immobilization.[10] However, they have some disadvantages: low moldability, compaction under high pressure, and low stability in high ionic concentrated solution or phosphate buffer solution. Consequently, it is important to develop a matrix that is more stable than conventional matrices.

Kurokawa (an author of this article) has reported that a compatible composite gel fiber was

Correspondence to: K. Nakane.

Journal of Applied Polymer Science, Vol. 81, 2084–2088 (2001)

formed between cellulose acetate (CA) and titanium alkoxide.[11] It was shown that this gel fiber was useful for an enzyme entrap-immobilization matrix.[12–14] In the current study we examined the preparation by gel formation of a composite gel fiber (CA–ZrO_2 fiber) from CA and zirconium alkoxide and the entrap-immobilization of invertase on this gel fiber. Among many metals the element zirconium has excellent vital stability. It is said that intermixing metals such as Al^{3+} and Zn^{2+} into blood through food is harmful for nerve endings. Therefore, these metal oxides are not suitable in practice as the support matrix of immobilization.

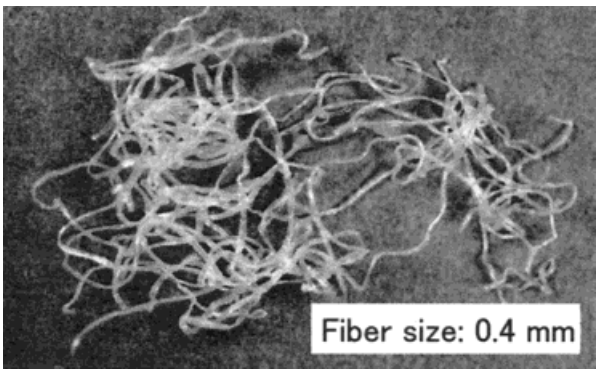

Figure 1 A view of CA–ZrO_2 composite gel fiber.

EXPERIMENTAL

Materials

All reagents were commercially obtained and of reagent grade. Invertase (EC 3.2.1.26, from bakers' yeast) was purchased from Sigma Chemical Company, U.S.A. Cellulose acetate (CA; M_w = ca. 45,000), with a 39.8% acetyl content, was obtained from Wako Pure Chemicals Ind., Ltd., Japan. Zirconium tetra-*n*-butoxide and titanium isopropoxide were obtained from Kanto Chemical Company, Inc., Japan, and Wako Pure Chemicals Company, Japan, respectively. Prior to the experiments, acetone was dehydrated using molecular sieves (3A 1/16, Wako Pure Chemicals Ind., Ltd., Japan).

Preparation of Entrap-Immobilized Invertase

Placed into a syringe was 10 wt % CA acetone solution (spinning solution) in which invertase had been dispersed (3 wt % g-CA). This was extruded into a stirred 5 wt % zirconium tetra-*n*-butoxide (or titanium isopropoxide) acetone solution (coagulation solution bath) through a needle with compressed N_2 gas of 2 atm. The needle tip was placed 2 cm above the surface of bath. The CA–ZrO_2 (or TiO_2) gel fiber was formed by this procedure. After standing for 30 min, the resulting fiber was removed from the solution. It was then washed with acetone and distilled water several times to remove the residual alkoxide. It was stored overnight in acetate buffer (pH 5.5) at 5°C. The size of the fiber was changeable from 0.4 mm to 1.0 mm by adjusting the needle diameter.

Alginate beads (ca. 3 mm in diameter) immobilized with invertase were obtained by the conventional procedure. Added dropwise to 1 wt % aqueous solution of calcium chloride (Nacalai Tesque, Inc., Japan) was 1 wt % aqueous solution of sodium alginate (Nacalai Tesque, Inc., Japan) containing invertase.

Evaluation of Activity

Activity of invertase was estimated by determination of product glucose using a Glucose B-Test Wako Kit (Wako Pure Chemicals Ind., Ltd., Japan).

The main reaction condition was as follows: 3 mL of invertase solution (1 g/L) was added to a mixture of 30 mL of sucrose solution [pH 5.5 (acetate buffer)]. Also, 0.2 g of immobilized invertase was added to a 30-mL mixture of sucrose solution. The actual quantities of native and immobilized invertase (CA–ZrO_2 fiber) were 3.0×10^{-3} and 2.2×10^{-3} g, respectively. They were reacted at 37°C for 15 min. Parameters such as pH (3.5–9.0), temperature (30–80°C), and concentration (0.01–0.13 mol/L) were changed for each purpose. The buffer solutions used were: acetate for pH 3.5–5.5, phosphate for pH 6.5–7.5, and borate for pH 8.5. A 0.02-mL aliquot was taken from the reaction solution and added to 3 mL of the Wako Kit solution and color-developed. The absorbance was measured at 505 nm using a Shimadzu UV-120.

RESULTS AND DISCUSSION

Characteristics of CA–ZrO_2 Fiber

Figure 1 shows a view of the resulting gel fiber, which is semitransparent and slightly elastic in water. It has a smooth surface with an absence of large pores, as shown by SEM (scanning electron

Table I Stabilities of Alginate–Ca Beads and CA–ZrO_2 Fibers in Various Aqueous Solutions (Gel Immersed in Solution at 37°C for 2 Days)

Solution	Support Gel	
	Alginate–Ca beads	CA–ZrO_2 fiber
0.1*M* KCl	Swell	No change
1*M* KCl	Highly swell	No change
0.1*M* NaCl	Swell	No change
1*M* NaCl	Highly swell	No change
0.1*M* Phosphate buffer (pH 6.0)	Swell	No change
0.1*M* Phosphate buffer (pH 7.0)	Swell	No change
0.1*M* Phosphate buffer (pH 8.0)	Swell	No change

Swollen gels do not keep the original bead forms.

microscope) observation. The fiber is considered to be formed by coordinated interaction between the zirconium and the oxygen of the hydroxyl group or the carbonyl group on CA molecules.[15] It is probable that invertase must be entrap-immobilized in the three-dimensional network of CA–ZrO_2 fibers.

The stabilities of the CA–ZrO_2 fiber and alginate gel beads in various solutions are compared in Table I. They were immersed in aqueous solutions at 37°C for 2 days. Alginate gel was swollen in each solution, according to circumstance, losing bead forms. This phenomenon is represented by "highly swell" in Table I. This may be because of the release of Ca^{2+} from gel by the exchanging of Ca^{2+} in the alginate gel with Na^+ in solution. Fiber is not swollen and is more resistant against ionic solution. There is no change of fiber in these ionic solutions, indicating that the coordination of cellulose on ZrO_2 is strong. About 10 wt % ZrO_2 per g-fiber remained after calcination of the fiber at 600°C for 8 h.

Characteristics of Immobilized Invertase

The effect of enzyme loading on activity was examined. Activity increased linearly with enzyme concentration in the CA–ZrO_2 fiber, indicating that invertase was dispersed homogeneously within gel fiber. The gel fiber without invertase did not show any activities. The following experiments were performed for the fiber containing 3 wt % enzyme (the spinning became difficult with greater invertase loading because the needles were choked).

The durability of the immobilized enzyme is important in applications because it is subjected to repeated reactions. Figure 2 shows the effect of repeated use on the residual activity by immobilized invertase. The reaction (15 min) was repeated on the same fiber at intervals of 10 min. The immobilized invertase was washed with buffer after each reaction and then immersed again in a fresh reaction solution. About 92% of the initial activity held after cycling 10 times for the CA–ZrO_2 fiber. This suggests that a significant leakage of invertase from fiber does not occur under repeated washings. On the other hand, a drastic decrease of activity was observed after repeated uses of immobilized alginate beads. Such deterioration with use was observed for the alginate beads, but not for the fiber.

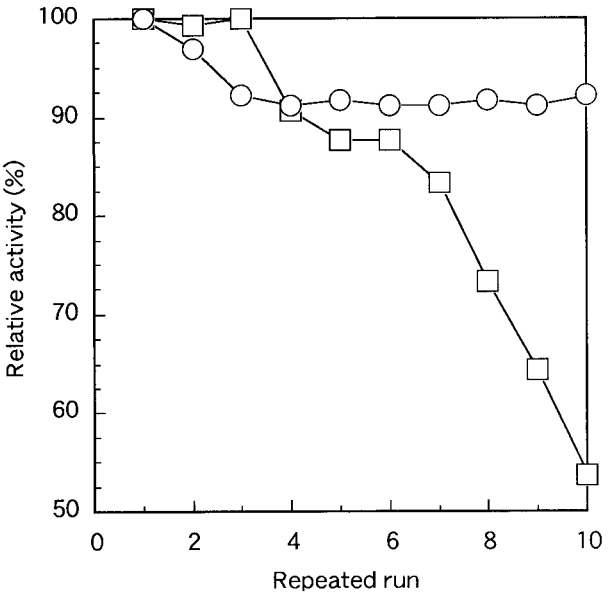

Figure 2 Effect of repeated run on relative activity of immobilized invertase. Initial activity was adopted as 100%. [(○) CA–ZrO_2 fiber, (□) alginate; condition: pH 5.5, temperature 37°C].

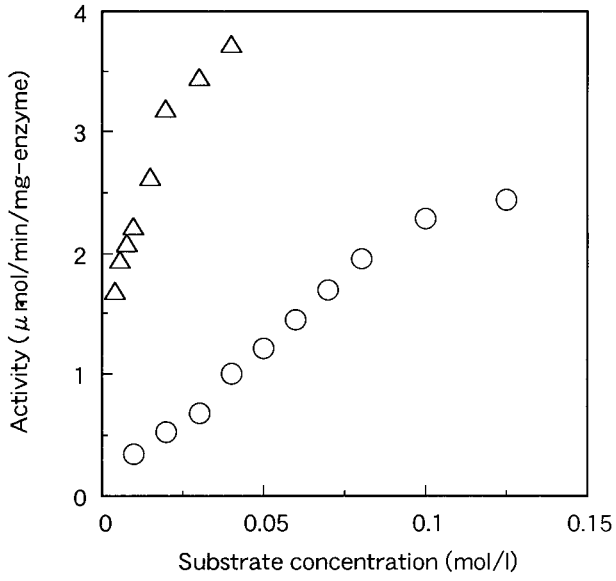

Figure 3 Effect of substrate concentration on activity: (△) native, (○) immobilized (CA–ZrO_2 fiber).

Figure 3 shows the effect of substrate concentration on activity. Activity increases linearly up to 0.02*M* for native enzyme and to 0.08*M* for immobilized enzyme. Applicability of the Michaelis–Menten law was confirmed in the low concentration range by the Eadie–Hofstee plot. The plot gave a straight line, characteristic of the reaction obeying the law. The apparent Michaelis constant (K_m) and maximum reaction velocity (V_{max}), which were obtained from the slope and the intercept of the straight line, are given in Table II. The apparent K_m value is much higher for immobilized invertase than for the native one, while the V_{max} values of immobilized invertase are smaller than those of native invertase. The higher K_m means less affinity of the substrate for immobilized enzyme. It seems that the structure of the CA–ZrO_2 fiber is not porous, a situation in which the substrate does not diffuse easily into the fiber. Thus, an enzyme entrapped inside a gel fiber does not participate in the reactions, while an enzyme entrapped on the surface of the fiber is involved in the reaction.

Table II Apparent Michaelis Constant (K_m) and Maximum Reaction Velocity (V_{max}) of Native and Immobilized Invertase

Invertase	K_m (mol/L)	V_{max} (mol/min/g-enzyme)
Native	2.70×10^{-3}	2.82×10^{-3}
Immobilized (CA–ZrO_2 fiber)	2.22×10^{-2}	1.17×10^{-3}

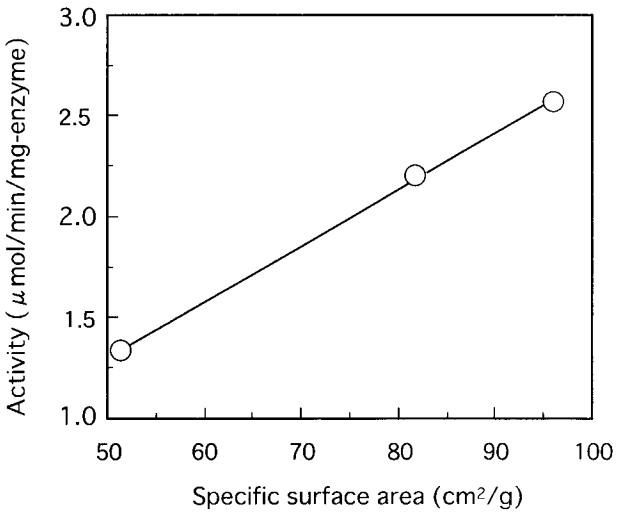

Figure 4 Effect of specific surface area of CA–ZrO_2 fiber on activity.

Figure 4 shows the relationship between the specific surface area and the activity (substrate concentration: 0.01*M*) of the fiber. The specific surface areas were calculated by assuming that the fiber is a cylinder that has a smooth surface. As seen from the figure, a linear relationship can

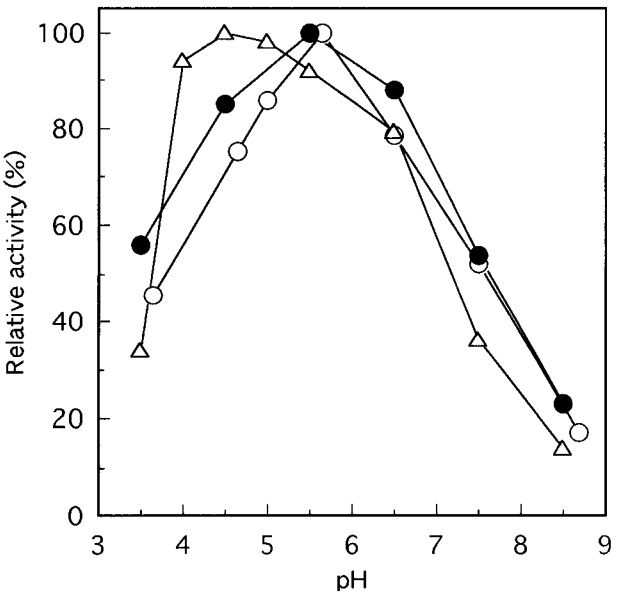

Figure 5 Effect of pH on activity of native and immobilized invertase. The optimal pH was adopted as 100% [(●) immobilized (CA–ZrO_2 fiber), (○) immobilized (CA–TiO_2 fiber), (△) native].

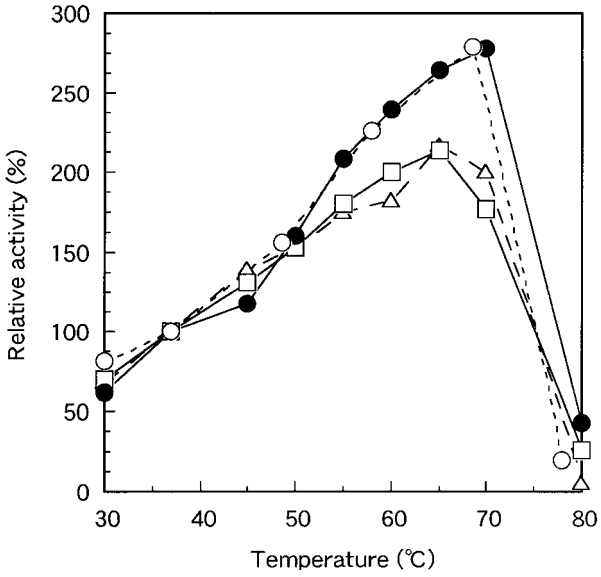

Figure 6 Effect of reaction temperature on activity. Activity at 37°C was adopted as 100% [(○) immobilized (CA–ZrO_2 fiber), (●) immobilized (CA–TiO_2 fiber), (△) native, (□) immobilized (alginate beads)].

be observed. This result suggests that the enzyme reaction occurs in the neighborhood of the fiber surface, as indicated by a lower V_{max}.

The pH and the thermal stabilities of invertase were changed by the entrap-immobilization on CA–ZrO_2 fiber. Figure 5 shows the pH profiles of activity relative to that at pH 5.5 and 37°C. The result of CA–TiO_2 gel fiber is also shown in this figure. Optimal pH is observed at around 5.0 and 5.5 for native and immobilized invertase, respectively. The immobilized invertase displays a greater activity at higher pH values. A similar tendency can be observed for the CA–TiO_2 fiber. It seems that the pH in the fiber is different from that in bulk solution because the ZrO_2 and TiO_2 in fibers denote an amphoteric ion exchange property—acting as both an anion exchanger and a cation exchanger depending on the pH of the solution.

The effect of the reaction temperature on the activity is shown in Figure 6. Both immobilized invertases are more active in a higher temperature range, as shown in this figure. This may be because of the prevention of thermal denaturation by rigid immobilization. An enhancement of thermal stability is not found in immobilized invertase on the alginate beads. This may be a result of the soft immobilization on alginate gel, in which the invertase is liable to undergo thermal denaturation.

CONCLUSIONS

Invertase was entrap-immobilized on a composite gel fiber of cellulose and hydrous zirconium oxide prepared by gel formation. The immobilization procedure was very simple and easily handled. Invertase was immobilized favorably on the gel fiber. The activity of the immobilized invertase became higher with increasing fiber diameter, indicating that hydrolysis of sucrose occurs in the neighborhood of the fiber surface. The pH maximum of activity for immobilized invertase shifted to the more alkaline side as compared to native invertase, reflecting the amphoteric property of ZrO_2.

REFERENCES

1. Veliky, I. A.; McLear, R. J. C. Immobilized Biosystems; Blakie Academic & Professional: New York, 1994.
2. Boudrant, J.; Cheftel, C. Biotechnol Bioeng 1975, 17, 827.
3. Kierstan, M. Biotechnol Bioeng 1977, 19, 387.
4. Sato, T.; Takamatsu, S.; Chibata, I. Enzyme Eng 1982, 6, 271.
5. Queiroz, A. A. A.; Vitolo, M.; Oliveira, R. C.; Higa, O. Z. Radiat Phys Chem 1996, 47, 873.
6. Emregui, E.; Sungur, S.; Akbulut, U. Biomaterials 1996, 17, 1423.
7. Uhlich, T.; Ulbricht, M.; Tomaschewski, G. Enzyme Microb Technol 1996, 19, 124.
8. Kizilyar, N.; Akbulut, U.; Toppare, L.; Ozden, M. Y.; Yagci, Y. Synthetic Metals 1999, 104, 45.
9. Yoshii, H.; Koyama, Y.; Obana, H.; Hikuma, M.; Yoshimura, M.; Yokozeki, K.; Ikeda, S. Nippon Nogeikagaku Kaishi 1993, 67, 1271 (in Japanese).
10. Immobilized Biocatalyst; Chibata, T., Ed.; Kodansha: Tokyo, 1990 (in Japanese).
11. Kurokawa, Y.; Ohta, Y. Kobunshi Kako (Polymer Applications) 1992, 41, 398 (in Japanese).
12. Ohmori, Y.; Kurokawa, Y. J. Biotech 1994, 33, 205.
13. Kurokawa, Y.; Sato, T. J Mater Sci Lett 1994, 13, 516
14. Kurokawa, Y.; Kobayashi, S.; Gamou, Y. Hyomen (Surface) 1998, 36, 382 (in Japanese).
15. Nakane, K.; Takahashi, K.; Suzuki, F.; Kurokawa, Y. Sen-i Gakkaishi 1999, 55, 563.