COMMUNICATION TO THE EDITOR

Adsorption and Enzyme (β-galactosidase and α-chymotrypsin): Immobilization Properties of Gel Fiber Prepared by the Gel Formation of Cellulose Acetate and Titanium Iso-Propoxide

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Abstract: We prepared a new composite gel fiber by the gel formation of cellulose acetate and titanium iso-propoxide. The fiber is harder than alginate gel; it is also stable in common solvents, phosphate solution, and electrolyte solutions over a wide range of pH from 3 to 10. The fiber shows amphoretic adsorption properties depending on pH, namely, it acts anionic with decreasing pH and cationic with increasing pH. However, the fiber had no adsorption property for a pyrogen endotoxin. The β-galactosidase and α-chymotrypsin not retained in alginate gel were immobilized on the fibers by this method. The pH, temperature, and repeated run stabilities of the immobilized enzyme were compared to those of the native one. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* **59:** 651–656, 1998.

Keywords: cellulose; gel; fiber; immobilization; adsorption

INTRODUCTION

There have been many studies on enzyme-immobilization (Veliky and McLean, 1994). Although interest in the use of cellulose as a matrix for immobilizing enzymes has existed for a long time, cellulose has not been applied to a significant degree (Gemeiner et al., 1993). There are many studies of the immobilization on cellulose by a covalent-bonding method, but few by enzyme-immobilization by entrapment because it is not easy to dissolve cellulose in aqueous solution. Cellulose is among the most abundant renewable organic resources, which is also easily biodegradable thus less contaminating to the environment. The enzyme-immobilization by entrapment using an alginate gel is fast, mild, and low-cost, which may be used with a wide range of biocatalysts (Veliky and McLean, 1994). However, this method has some disadvantages. Alginate gel swells in high concentrations of K^+ and Mg^{2+} ions, phosphate and chelating agent solutions; in fact, it may be dissolved in these solutions. The gel network is so soft that a low molecular weight enzyme such as protease leaks out of the gel network. Carrageenan has properties similar to agarose and alginate. However, it is not used as frequently as alginate. Due to the softness of their gels, they show compaction and a large pressure drop in a flow-packed column. Inorganic matrices such as SiO_2, Al_2O_3 powders and ceramics have been used as the immobilization matrices (Veliky and McLean, 1994). They show no compaction or large pressure drop due to their rigidities. However, they dissolve in lower or higher pH solutions. Hydrous TiO_2 and ZrO_2 have been shown to be suitable as immobilization matrices (Rogalski et al., 1994; White and Kennedy, 1980). The immobilization is performed by hydrolysis of a $TiCl_4$ solution coexisting with the enzyme. However, hydrous TiO_2 is very fine, and a finite amount of enzyme leaks out from the matrix by washing or during reactions. In a previous report, the authors reported a new enzyme-immobilization by entrapment of an enzyme on a cellulose-polyvalent metal (Ti, Zr) alkoxide gel fiber (Kurokawa, 1996). Here, we report the adsorption properties of the fiber for inorganic ions and endotoxin, and the immobilization of β-galactosidase or α-chymotrypsin on it.

EXPERIMENTAL

All reagents were commercially obtained and of reagent grade. Cellulose acetate (CA) is commercially obtained from Wako Pure Chemicals (Osaka 540, Japan) and has a 39.8% acetyl content. Prior to the experiments, reagent grade acetone was dehydrated using molecular sieves (3Å1/16, Nakalai Tesque Inc., Kyoto 604, Japan).

The 10 wt% cellulose acetate acetone solution (spinning

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solution) in which the enzyme (3 wt% for CA) was dispersed, was extruded into a stirred 10 wt% titanium isopropoxide acetone solution (coagulation solution bath) through a nozzle with N_2 compressed gas. The nozzle tip was placed just beneath the bath surface. After standing for 30 min, the resultant fiber was removed from the solution. It was then washed with acetone and water several times to remove the residual alkoxide. It was stored at 5°C until use. For the adsorption gel fiber, the spinning solution without enzyme was slowly fed through the coagulation bath. After standing, the gel fiber was washed with water and dried at room temperature.

The adsorption of K^+ or phosphate ion on the fiber was studied as a function of pH. One gram of dried fiber was added to 100 mL of a 0.1*M* test solution adjusted by adding 0.1*M* HCl or 0.1*M* NaOH solutions. The samples were equilibrated at ±0.1°C for one week. Calibration test solutions were prepared from $NaH_2PO_4 \cdot 2H_2O$ and KCl, respectively. These ions were determined by an ICP Atomic Emission Spectrophotometer (Shimadzu, ICPQ-100) and an Atomic Adsorption Spectrophotometer (Hitachi). Endotoxin (*E. Coli.* 055:B5) was obtained from Seikagaku Co. The endotoxin concentration was assayed by the Seikagaku Toxicolor System LS-200. Adsorptions were carried out for aqueous solutions of pH 7 and 8 adjusted with phosphate buffer and for an aqueous solution using pure water.

The β-galactosidase (E.C. 3.2.1.23) from *asparagillus oryzaea* and α-chymotrypsin (E.C. 3.4.21.1) from bovine pancreas were obtained from Sigma Chemical Co., and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and lactose were from Nakarai Tesque Inc.

The activities of β-galactosidase were assayed by its incubation with lactose and ONPG substrates (Bakken et al., 1991; Ovsejevi et al., 1995). Measurements were made on solutions (10 ~ 20 mL) ranging in substrate concentration from 0.01 to 0.15 mol/L. Enzyme concentration is 1 mg/mL for the native reaction and 3 wt% fiber for immobilized reaction. The enzyme reaction (ONPG) was carried out for 15 min and stopped by adding 1 mL of 1*M* Na_2CO_3 to the reaction mixture. The amount of released *o*-nitrophenol was determined spectrophotometrically by measuring the absorbance at 420 nm. The lactose reaction was evaluated with a Glucose D-Test Wako Kit. Color-producing reagent was added to the reaction solution. The produced color (equivalent to produced glucose) was determined spectrophotometrically at 505 nm. The α-chymotrypsin activity was evaluated by hydrolysis of the *N*-succinyl-*L*-phenylalanine-*p*-nitroanilide substrate (SPNA) (Bergmeyer, 1983). The enzyme reaction was carried out for 30 min. The enzyme concentration was 5 mg for the native reaction (10 mL) and 0.5 g fiber for the immobilized reaction (25 mL). The product *p*-nitroaniline was determined spectrophotometrically at 405 nm. A Shimadzu UV-2200 spectrophotometer was used in all measurements. Continuous reaction of lactose by the immobilized enzyme was performed at 37°C. Small amounts of samples were withdrawn from the reaction vessel (450 mL) at regular time intervals, cooled to room temperature, and analyzed for residual activity.

The reusability of the immobilized enzyme was determined using the same enzyme for a number of cycles after washing it thoroughly each time. The pH stability of the enzyme was determined by measuring the activity of the enzyme at various pH values. The reaction time, temperature, and substrate concentration were 15 min, 37°C, and 0.05*M* for β-galactosidase. They were 30 min, 25°C, and 4 m*M* for α-chymotrypsin. The buffer solutions used were phosphate-citrate for pH 3 ~ 7, tris, and phosphate for pH 7.5 ~ 9.0.

For the determination of the optimum temperature, the native and immobilized enzymes were incubated with the substrate at various temperatures for 15 min at the optimum pH (4.5 for β-galactosidase, and 7.8 for α-chymotrypsin).

The thermal stability of the immobilized enzyme was evaluated by measuring the residual activity of the enzyme exposed at various temperatures for 24 h; the sample was quickly cooled at 30°C and assayed. A jacketed reaction glass vessel (450 mL) was used as a continuous flow reactor for lactose hydrolysis.

RESULTS AND DISCUSSION

The resultant gel fiber is slightly elastic and transparent. It shows no X-ray diffraction pattern, indicating homogeneous and amorphous properties. It also contains 13–15 wt% TiO_2 and 10–15 wt% water, depending on the preparation conditions. It has a diameter of 0.5 ~ 0.7 mm. The stabilities in various solutions are compared in Table I for alginate beads, carrageenan beads, and CA-TiO_2 fibers. They were not treated with a cross-linking agent such as glutaldehyde. They were immersed in the solutions at 37°C for 2 d. Alginate and carrageenan beads were swollen in higher ionic and phosphate solutions, under certain conditions, losing their bead form. The lack of resistance to phosphate ion is a serious problem in the reaction of the immobilized cell. Carrageenan gel has properties very similar to those of agarose and alginate gels.

Table I. Stabilities of alginate, κ-carrageenan, and Ti-CA gels at 37°C for 2 d.

	Gel species		
Aqueous solution	Alginate-Ca bead	κ-Carrageenan bead	Ti-CA fiber
0.1*M* KCl	Swell	a*	No change
1.0*M* KCl	Highly swell	a	No change
0.1*M* NaCl	Swell	Dissolution	No change
1.0*M* NaCl	Highly swell	No change	No change
0.1*M* $CaCl_2$	a	No change	No change
1.0*M* $CaCl_2$	a	No change	No change
pH 6.0 phosphate buffer	Highly swell	Dissolution	No change
pH 7.0 phosphate buffer	Swell	Dissolution	No change
pH 8.0 phosphate buffer	Swell	Dissolution	No change

*a: Gelation medium.

The difference in alginate, carrageenan gels, and CA-TiO_2 gel may be due to the fact that the former gel formations are an ionic reaction of the cation with COO^- or SO_3^{2-} on the pyranose ring of cellulose, while the latter is a coordinative reaction of the polyvalent cation such as Ti or Zr with ligands such as OH or CO functions on pyranose ring.

The gel fiber has a smooth surface with an absence of large pores similar to alginate gel. Figure 1 shows the adsorption profile for ionic species. The adsorption of phosphate is represented by the total amount of phosphate as a unit of P_2O_5 because phosphate takes some species in aqueous solution. The uptake of phosphate increases with a decrease in pH, and that of K^+ increases with an increase in pH. CA film does not adsorb the phosphate as shown in the figure. The pH at the point of intersection approximately coincides with the isoelectric point, 6.0, which slightly depends on the condition of the hydrous TiO_2 crystallization. This indicates that the amphoteric properties of the TiO_2 gel acting both as an anion and cation exchanger are dependent on the pH of the solution. The pyrogen, endotoxin, is a lipopolysaccharide containing a highly hydrophobic region, lipid A, in its structure. Some attempts have been made to remove the endotoxin from the final bioproducts (Morimoto et al., 1995). Hydrophobic and electrical interactions are considered as major forces for endotoxin adsorption. The gel fiber has a hydrophobic cellulose and an electrically charged TiO_2. The authors tried to adsorb the endotoxin onto the fiber. However, the fiber did not adsorb the endotoxin from the aqueous solution as shown in Table II. This may be due to the nonporous surface on the fiber. It has a smooth surface without large pores, which has a low surface area (<10 m^2/g). Otherwise, the surface may not be hydrophobic to the endotoxin as expected.

A number of researchers have investigated the hydrolysis of lactose by immobilized β-galactosidase on porous glass, charcoal, chitosan, and ion-exchange resin prepared by covalently coupled and adsorbed methods (Bakken et al., 1991; Ovsejevi et al., 1995; Rejikumar and Devi, 1995; Saito et al., 1994). In enzyme-immobilization by entrapment, much of the activity is lost due to leakage from the alginate gel. It has been shown that the globular protein, α-chymotrypsin, is not immobilized in an alginate gel (Reischwitz et al., 1995).

Table II. Adsorption of endotoxin on gel fiber at 25°C.

Endotoxin solution	Initial concentration (ng/mL)	Residual concentration (ng/mL)
No buffer condition	450	450
pH 7 phosphate buffer	470	450
pH 6 phosphate buffer	450	450

It is also shown that inulin of a molecular weight of 3–5 $\times 10^3$ is not retained on alginate gel. The immobilized β-galactosidase is of interest from a nutritional standpoint in reducing lactose, while the immobilized α-chymotrypsin is of interest for both the synthesis of oligopeptide and the treatment of cheese whey in the dairy industry. While the hydrolytic activity of β-galactosidase has been extensively studied, by comparison, its transferase activity has been neglected. Recently, however, there has been renewed interest in the transference reaction. Here, we are interested in whether this sol-gel method can be applicable to both enzymes previously described. Therefore, experiments were performed using the two substrates lactose and ONPG in low concentration for β-galactosidase and SPNA for α-chymotrypsin. Casein was not used because its molecular weight varied widely from source-to-source.

The durability of the immobilized enzyme is important in applications because it is subjected to repeated reactions. The immobilized enzyme was washed and then suspended again in a fresh reaction solution. The cycles were repeated on the same fiber every 10 min. Figure 2 shows the effect of repeated use on the residual relative activity. The relative

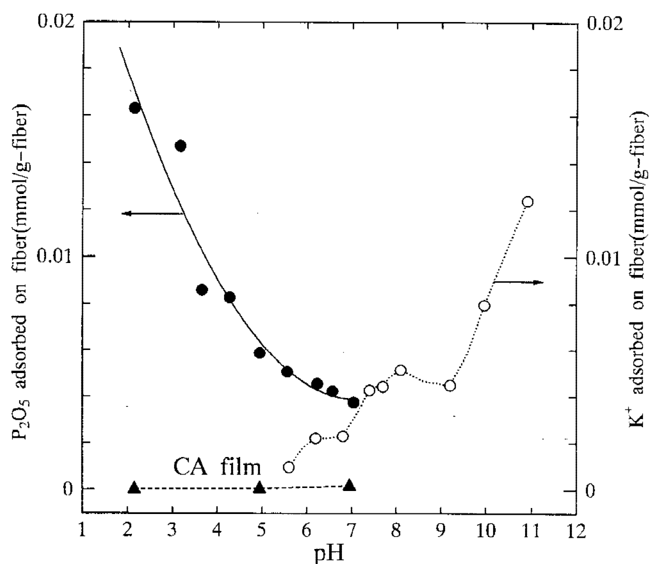

Figure 1. Adsorption of K^+ and phosphate on CA-TiO_2 gel fiber and CA film as a function of pH of aqueous solution.

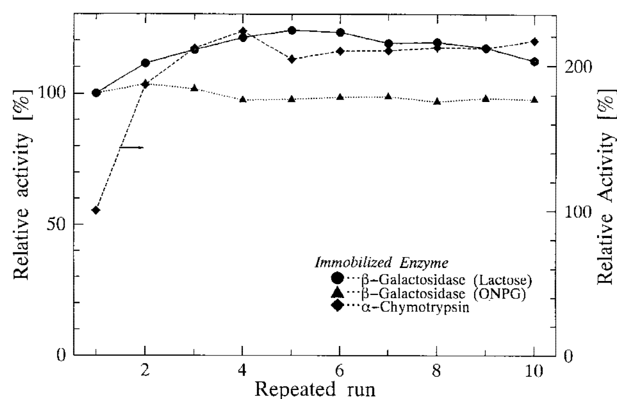

Figure 2. Effect of repeated runs on the residual activities. Initial activity is 100%.

activity was calculated under the assumption that the enzyme sustains the initial activity. The activity is seen to be retained without any definite loss, even if the batch reactions are repeated at least 10 times. It is found that the activities of immobilized β-galactosidase for ONPG and lactose are found to be equivalent to the initial values, suggesting that significant leakage of the enzyme from the fiber did not occur during the repeated washings. However, the activity of immobilized α-chymotrypsin increases with the initial repeatings and reaches a steady state. It may take a long time to establish a stable conformation of the enzyme in the reaction path. Because the globular protein, α-chymotrypsin, has a low molecular weight (≈25,000), it is reported that this enzyme is not immobilized in an alginate gel (Reischwitz et al., 1995). Alginate gel immobilization is mild and simple, which may be applied with a wide range of biocatalysts. However, the gel network is so loose that the lower molecular weight enzyme leaks out. The protease can be immobilized on this gel fiber as shown in Figure 2. This may be due to hard physical entrapment in CA-TiO_2 gel and/or to the chelating properties of the titanium ion, which can be employed to couple the enzyme. The enzyme has amine and carboxyl groups. These are considered to act as ligands toward titanium. In view of these considerations, hydrous TiO_2 is expected to exhibit general affinity for the binding of other proteins.

The effects of temperature on the activities are shown in Figure 3. Immobilized β-galactosidase has the same optimum temperature (≈55°C) as the native enzyme. At a higher temperature than this, the activities rapidly decreased due to the denaturation of the enzyme. The optimum temperature for immobilized α-chymotrypsin is lower than that of the native enzyme. This indicates that the active site of the enzyme is affected by immobilization. This may reflect an initial increase in activities due to an unstable conformation as shown in Figure 3. This may be ascribed to harder immobilization compared to the alginate gel.

The thermal stability is also a very important parameter as part of the optimum temperature. Figure 4 shows the thermal stability data. Unexpected trends are observed in

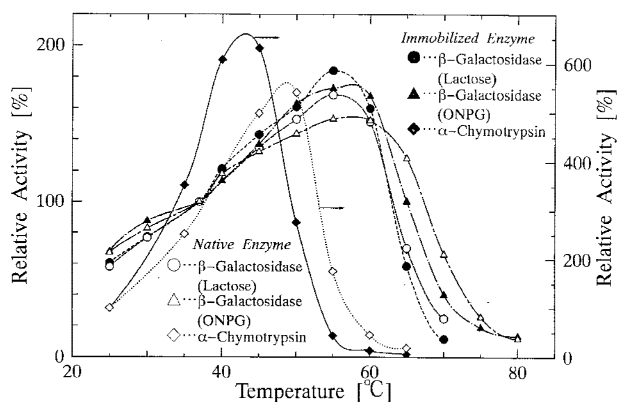

Figure 3. Effect of temperature on activities of native and immobilized enzymes (β-galactosidase and α-chymotrypsin). Activity at 37°C is 100% for β-galactosidase and that at 25°C is 100% for α-chymotrypsin.

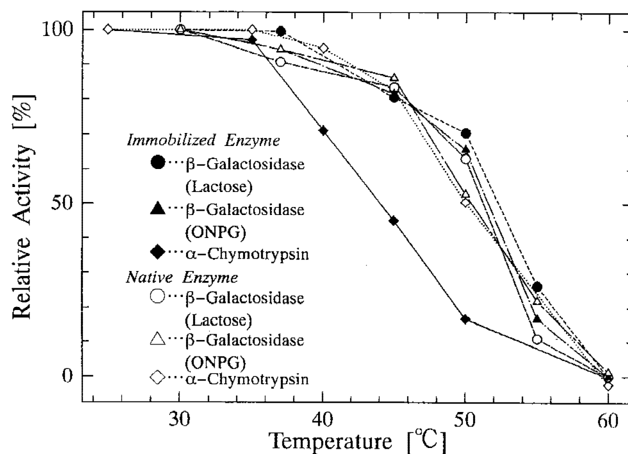

Figure 4. Thermal stabilities of native and immobilized enzymes at the given temperature on the residual activities. Incubation time: 1 h for α-chymotrypsin, 24 h for β-galactosidase. Activity at 30°C is 100% for β-galactosidase and at 37°C is 100% for α-chymotrypsin.

that no significant change in temperature effect is observed between immobilized and native enzymes, and the immobilized α-chymotrypsin is less stable than the native one in the range of higher temperature. This indicates that β-galactosidase is conformationally not affected, but α-chymotrypsin is affected by immobilization. Further, as shown in Figures 2–4, there is no significant difference between the lactose and ONPG activities. This may imply that the transferase reaction (Smart, 1991; Toba et al., 1985) essentially does not occur at this low lactose concentration.

The plot of rate vs. substrate concentration gave a curve of the Michaelis-Menten type. The Michaelis-Menten constants are given in Table III. The apparent K_m values are greater for the immobilized than for the native enzyme, while the V_m values are smaller for the immobilized enzyme. The higher K_m value of the immobilized enzyme is probably due to a conformation change in the active site or lower affinity substrate for the gel fiber matrix. The V_m values of the immobilized enzymes are smaller than those of the natives. This may be due to greater rigidity of the gel, which limits the substrate diffusion in the matrix toward enzyme reaction sites, and due to a possible deactivation of the enzyme which has been occurring during the gel fiber formation by the close contact of the enzyme with acetone. Anyhow, enzyme reaction may occur in the vicinity of the

Table III. Michaelis constants for native and immobilized enzymes.

Enzyme	Substrate		K_m (mol/L)	V_{max} (mol/min/g-enzyme)
β-Galactosidase	ONPG	native	1.38×10^{-2}	8.44×10^{-3}
		fiber	4.55×10^{-2}	7.82×10^{-4}
	Lactose	native	0.93×10^{-1}	8.86×10^{-3}
		fiber	1.62×10^{-1}	2.89×10^{-3}
α-Chymotrypsin	SPNA	native	1.20×10^{-3}	5.48×10^{-3}
		fiber	2.08×10^{-3}	5.40×10^{-3}

fiber surface and does not easily occur in the interior. However, micro ions such as K^+ may diffuse into the matrix to a certain extent.

The pH profile is shown in Figure 5. The profile for an immobilized enzyme is widened because of diffusional limitations. The optimum pH for β-galactosidase shifts toward a more acidic pH value. This may be due to the amphoteric properties of the TiO_2 previously described. The pH dependence of α-chymotrypsin was performed at pH 8–9 because the substrate is not easily dissolved in water. The highest activity of α-chymotrypsin is reported to be at pH 8.5 (Riccardo et al., 1976). However, the activity is almost independent of pH in the range of 8–9. The immobilized α-chymotrypsin shows a pH maximum at 8.4, which is close to the value previously described. It seems that the optimum pH emerges due to the ionic atmosphere around the immobilized matrix.

Figure 6 shows the long-time performance of immobilized β-galactosidase in a continuous flow reactor. It shows the remarkable reaction stability and good conversion rate during continuous reaction to nearly 40 d. Due to the structural rigidity of the gel fiber, no compaction and large pressure drop in the flow column occur.

The adsorption properties of the gel fibers prepared by the gel formation of cellulose and titanium iso-propoxide have been examined. Enzymes (β-galactosidase and α-chymotrypsin) have also been immobilized on the fiber. The gel fiber shows amphoteric properties depending on the content of TiO_2. Both enzymes have been favorably immobilized, but α-chymotrypsin has undergone a strained conformation due to the rigidity of the gel. However, the rigidity may be improved by a coexisting surface active reagent such as Triton X-100 in matrix. TiO_2 has a bio-suitability for enzymes as described in the Introduction. The most effective enzyme-immobilization property was obtained when the activated cellulose treated with aqueous $TiCl_4$ solution was employed as a matrix (Emery et al., 1972).

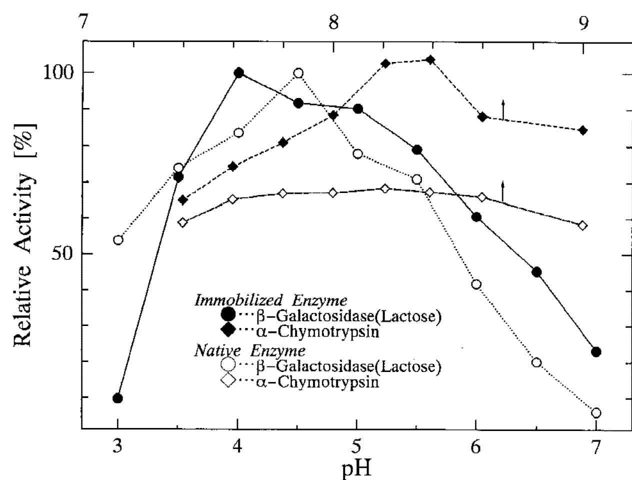

Figure 5. Effects of pH on activities of native and immobilized enzymes. The pH optimum is 100% except for native α-chymotrypsin (arbitrary unit). Temperature: 37°C. Concentration: 0.05*M*.

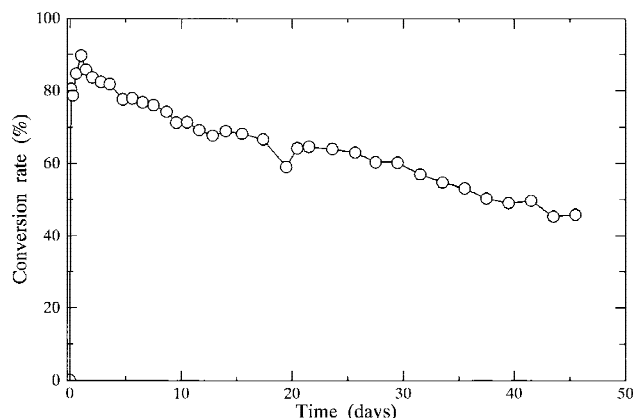

Figure 6. Continuous hydrolysis of lactose. Reactor volume: 450 mL. Lactose concentration: 0.04*M*. Temperature: 37°C. pH: 4.5. Fiber density: 7.5%. Flow rate: 15 mL/h.

Another advantage of introducing TiO_2 in CA is a higher resistance to compaction in a packed column compared to CA alone. The coordination between the $-COO^-$, $-NH_2$, and –SH groups of the enzyme and hydrous titanium oxide dispersed in CA may be favored. We are now investigating the interaction of amino acid with TiO_2 fine particles by IR and XPS measurements. Recently, there have been many reports on enzyme-immobilization by entrapment by a sol-gel process (Avnir et al., 1994). However, tetraethoxysilane (TEOS) is used exclusively for the hydrolyzed gel. In this composite gel, the hydrolysis of alkoxide and the coordination of the metal to CA or the enzyme may participate in the immobilization.

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