Synthesis of Geranyl Acetate by Lipase Entrap-Immobilized in Cellulose Acetate-TiO_2 Gel Fiber

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ABSTRACT: Lipase from *Candida antarctica* was entrap-immobilized in cellulose acetate-TiO_2 gel fiber (fiber-immobilized lipase) by the sol-gel method. Syntheses of geranyl acetate and citronellyl acetate catalyzed by the fiber-immobilized lipase were studied in heptane solution. Conversions reached 85% for geranyl acetate after 100 h, and 75% for citronellyl acetate after 80 h, and these values were almost identical to those for syntheses catalyzed by nonimmobilized lipase, although the reaction rate was decreased by immobilization. Compared to those of the nonimmoblized lipase and commercially available immobilized lipase (Novozyme 435), the activity of the fiber-immobilized lipase was not particularly affected by changes in reaction conditions, such as bulk water content or substrate concentration. The fiber-immobilized lipase retained a high level of activity after six repeated uses, and almost no enzyme leakage from fiber was observed. However, the reactivity of the fiber-immobilized lipase was depressed at higher temperature, presumably due to dehydration by thermal contraction of the gel fiber.

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KEY WORDS: Cellulose acetate, citronellyl acetate, entrap-immobilization, gel fiber, geranyl acetate, lipase, sol-gel method, terpene ester synthesis.

The use of lipase as a catalyst of esterification in an organic solvent has been well described (1,2). In its practical application, lipase immobilization on an appropriate matrix is needed. Few reviews exist for immobilization of lipase (3–5). Immobilization has advantages over use of soluble or native enzyme, e.g., reuse, simplicity of operation, and improvement of stability. Entrap-immobilization is suitable for industrial use because it is easily performed and the cost of support materials is low. Most of the conventional matrices, such as calcium alginate and κ-carrageenan gels, are hydrophilic and do not have particularly good mechanical strength. In conjunction with the recent increase in studies utilizing enzymatic biotransformations, there is thus need for new support materials for achieving individual reactions.

Terpene ester synthesis by lipase-catalyzed esterification has received much attention over the last two decades (6–10). Among the terpene esters synthesized by this method, the acetates of geraniol and citronellol are important flavor and fragrance compounds used in the food and cosmetic industries. Industrial production of terpene esters is accomplished *via* a nonspecific chemical process requiring complicated purification steps. In contrast, lipase-catalyzed esterification can be performed under more moderate conditions and yields a high-quality product.

The authors previously reported a novel sol-gel–derived entrap-immobilization method that uses gel formation of cellulose acetate with transition metal alkoxide (11,12). This gel did not dissolve in common organic solvents and resisted concentrated electrolyte solutions and phosphate buffer solution. The immobilized enzyme showed good stability for long operational runs, although its activity was depressed when entrap-immobilized. This paper deals with the esterification of terpene alcohols (geraniol and citronellol) with acetic acid using this sol-gel–derived immobilized lipase in heptane solution.

EXPERIMENTAL PROCEDURES

Materials. All reagents were of commercially available reagent grade. CHIRAZYM L-2, Lyo. (lipase B from *Candida antarctica*) was purchased from Boehringer Mannheim (Tokyo, Japan). Novozyme 435 (lipase B from *C. antarctica* immobilized on an acrylic resin) was a gift from Novo Nordisk Bioindustry Co. (Chiba, Japan). Cellulose acetate (CA) was obtained from Wako Pure Chemicals (Osaka, Japan) and had a 39.8% acetyl content. Before use, reagent-grade acetone was dehydrated using molecular sieves (3A 1/16; Nacalai Tesque Inc., Kyoto, Japan).

Lipase-immobilization in cellulose acetate-TiO_2 gel fiber. The 10 wt% CA acetone solution (spinning solution) in which the CHIRAZYM (7.5 wt% for CA) was dispersed was extruded into a stirred 10 wt% titanium isopropoxide solution in acetone (coagulation solution) through a glass nozzle with N_2 compressed gas. The nozzle tip was placed just beneath the surface of the coagulation solution. After standing for 1 h, the resultant fiber was removed from the solution. It was then washed with acetone and water several times to remove the residual alkoxide and stored at 5°C until use. In this procedure, 7.5 g of gel fiber was obtained from 20 g of CA solution containing 150 mg of CHIRAZYM, so it was calculated that 1.0 g of gel fiber contained 20 mg of lipase.

Esterification method. Esterification was carried out in 25-mL screw-capped glass tubes. Unless otherwise specified, native (i.e., unimmobilized, 0.01 g of CHIRAZYM), Novozyme (0.01 g), or fiber-immobilized lipase (fiber) (1.0 g) was added to

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10 mL of heptane solution containing 0.24 mol/L geraniol and 0.2 mol/L acetic acid, respectively. DL-Menthol (70 μL) was also added as an internal standard. In the case of citronellyl acetate synthesis, concentrations were changed to 0.1 mol/L citronellol, and 0.1 mol/L acetic acid and linallol (70 μL) were added as an internal standard. The solutions were shaken at 80 stroke/min in a thermostated water bath at 30°C, and aliquots were periodically withdrawn and analyzed using a gas chromatograph [Hitachi 663-50 type (flame-ionization detector, N_2 carrier gas): packed polyethylene glycol (PEG) 20 M stainless column (3 m); column temperature: 135°C; injection temperature: 250°C; detection temperature: 250°C]. A control experiment without lipase was also carried out in a similar way. Since the yields of products were less than 0.5%, nonenzymatic esterification was negligible under this reaction condition. The effect of water content in solvent on activity was determined with a reaction solution containing 1–10 μL of additional water. The effect of reaction cycle on activity was examined for the same lipase. One reaction period was 20 h. After each run, lipase was washed with fresh heptane. The weight loss of the fiber at various temperatures was examined by measuring the weight of the fiber before and after incubation for 3 h in heptane.

Observation of lipase leakage from matrices. Leakage of lipase from acrylic resin (Novozyme) and CA-TiO_2 gel fiber (fiber-immobilized lipase) into an aqueous solution was examined with reference to the studies of Hertzberg *et al.* (13). Novozyme (20 mg), fiber-immobilized lipase (0.8 g), or CA-TiO_2 gel fiber (0.8 g, no enzymes were contained) was added to 10 mL of water. The solution was shaken at 30°C, and 1-mL samples were periodically removed. In the case of Novozyme, these were filtered quickly over qualititative filter paper (No. 5C; Advantec Toyo Kaisha, Ltd., Tokyo, Japan) to remove suspended particles. Samples from reactions containing fiber-immobilized lipase or the control gel fiber lacking enzyme did not have suspended particulate matter and therefore were not filtered. Absorbance of the samples was then measured at 280 nm (Hitachi ultraviolet-visible spectrometer U-2010). After measurement, the samples were returned to the reactions. For Novozyme, the material collected during filtration was also returned.

RESULTS AND DISCUSSION

The gelation of cellulose acetate-titanium isopropoxide is attributed to a coordination of the polyvalent titanium species with carbonyl groups on CA (12). Most of the titanium isopropoxides were hydrolyzed by washing with water, but, based on the infrared spectrum of gel fiber, some of them remained and contributed to the coordination (spectrum not shown). The resultant gel fiber was elastic and had a diameter of 0.4–0.5 mm. From the results of thermogravimetric-differential thermal analysis, the fiber contained 50–60 wt% water and 20 wt% inorganic materials. Lipases were physically entrapped among the gel networks and distributed throughout the gel fiber (14).

In the lipase-catalyzed esterification reaction, the water content of the solvent affects not only the equilibrium position of the reaction but also the activity of the enzyme (15). Enzymes require a small amount of water in order to retain the conformation of their active sites in organic solvent. Water plays an important role in maintaining the stability and polarity of the active site, but an excessive amount of water leads to enzyme inactivation. Figure 1 shows the relative activities of native lipase and fiber-immobilized lipase for geranyl acetate synthesis in heptane with various amounts of additional water. Water was added to 10 mL of substrate solution. Each reaction was carried out for 5 h. Relative activity was represented on the basis of activity of the reaction with no additional water. The activity of native lipase followed a typical pattern, increasing at low water content until it reached a maximum value, then decreasing gradually. The maximum may indicate the amount of water required to confer the conformational flexibility needed for catalysis (1,16). The bulk water content did not influence the activity of fiber-immobilized lipase. This indicates that the enzyme is entrap-immobilized within the fiber and the amount of water around the enzyme is not substantially affected by bulk water.

The time courses of the syntheses of geranyl acetate and citronellyl acetate are shown in Figure 2. The amount of enzyme in fiber-immobilized lipase is about twice that in native lipase in the reaction solutions. Although the reaction rate by fiber-immobilized lipase decreased, the yields of geranyl acetate and citronellyl acetate with the catalyst reached 85 and 75% after 100 and 80 h incubation, respectively. This may have been due to the fact that substrates do not easily diffuse into the active site of lipase in gel fiber, and only the lipases near the fiber surface can catalyze the reaction. The surface of this gel fiber is smooth and nonporous. Indeed, the surface area is less than 1 m^2/g, as calculated by using the Brunauer-Emmett-Teller adsorption isotherm. Since the obtained yields, 85% for geranyl acetate and 75% for citronellyl acetate, were

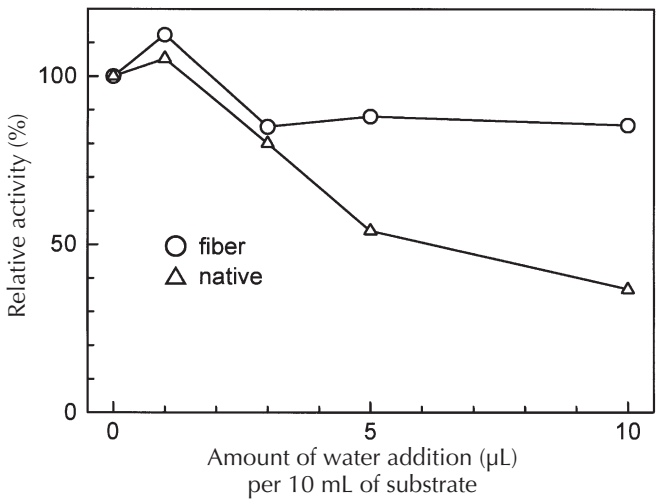

FIG. 1. Effect of water content in solvent on esterification of geraniol with acetic acid. Water (1, 3, 5, 10 μL) was added to 10 mL of substrate solution. Esterification was carried out in heptane at 30°C for 5 h.

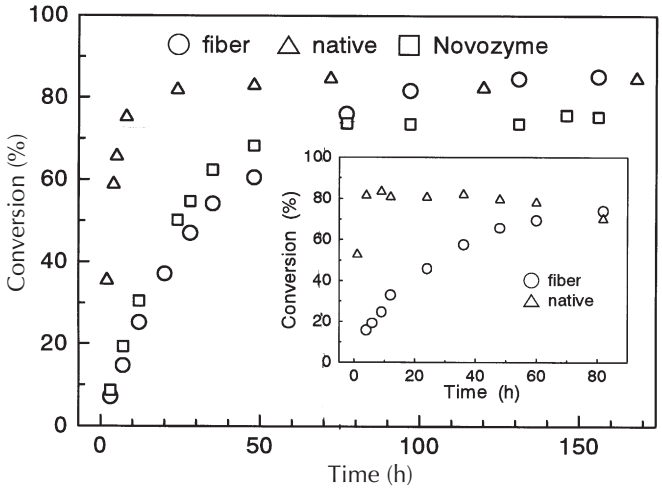

FIG. 2. Time course of lipase-catalyzed synthesis of geranyl acetate and citronellyl acetate (inner figure). Esterification was carried out in heptane at 30°C. Conversion was expressed relative to geranial and citronellol (inner figure). Novozyme (Novo Nordisk Bioindustry Co., Chiba, Japan).

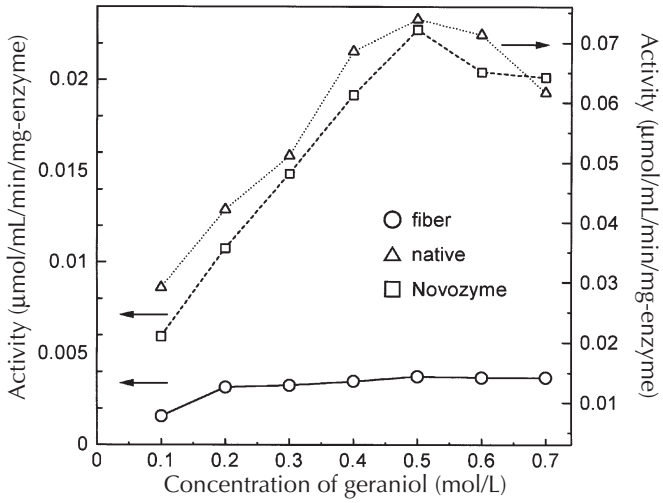

FIG. 3. Effect of concentration of geraniol on lipase-catalyzed synthesis of geranyl acetate at 30°C. Acetic acid was kept at a concentration of 0.2 mol/L, and geraniol concentration was varied. See Figure 2 for company source.

almost identical to those catalyzed by native lipase, it was considered that lipase entrap-immobilized in the vicinity of the fiber surface was able to maintain the activity during a long time reaction. An improvement in catalytic efficiency of fiber-immobilized lipase would be expected if the surface area of the fiber were increased by thinning the fiber or codoping with a surface-active reagent. Novozyme gave a 75% yield of geranyl acetate after 75 h incubation and the yield did not change until 155 h incubation. However, native or fiber-immobilized lipase gave higher yield (85%) in longer incubation. The lower yield given by Novozyme may be due to the shift of chemical equilibrium by adsorption of the produced water on porous matrix. On the other hand, it was supposed that the fiber-immobilized lipase did not adsorb water because the gel was nonporous. Although esterification of citronellol by Novozyme was not carried out in our study, it seemed that the yield of citronellyl acetate catalyzed by Novozyme should be lower than that catalyzed by native or fiber-immobilized lipase for the same reason as described.

Claon and Akoh (9) obtained a high yield (95%) of geranyl acetate using SP382 or SP435, which are *C. antarctica* lipases immobilized on acrylic resin by adsorption. They added molecular sieves in the reaction system in order to remove the water produced during the esterification. In the present study, the obtained yield was somewhat lower. This difference was probably because molecular sieves were not added to prevent a reverse reaction (hydrolysis). In an industrial-scale application, a reaction system without molecular sieves is considered desirable.

The effects of geraniol and acetic acid concentration on the esterification activities are shown in Figures 3 and 4, respectively. Activity was defined as the initial reaction rate per mg enzyme. In the case of Novozyme, activity is shown as the initial reaction rate per mg Novozyme. The activity of fiber-immobilized lipase was almost independent of the geraniol concentration, and as much as approximately one-seventh that of Novozyme. However, the esterification activities of native lipase and Novozyme were inhibited at higher concentration of geraniol (>0.5 mol/L). On the other hand, acetic acid inhibited the activities for all the lipases, as shown in Figure 4. The inhibitory effect of acetic acid has been reported previously (6–10), and it may be attributed to a lowering of pH in the vicinity of the enzyme. In addition, most lipases have little affinity for this two-carbon acid. Fiber-immobilized lipase showed high resistance to acetic acid inhibition, perhaps because acetic acid was not able to easily approach the enzyme entrap-immobilized in the inner surface of the gel fiber. This fact is consistent with the observed water-content dependence of activity (Fig. 1).

An improvement in the thermal stability of the enzyme can be expected due to immobilization. Figure 5 shows the effect

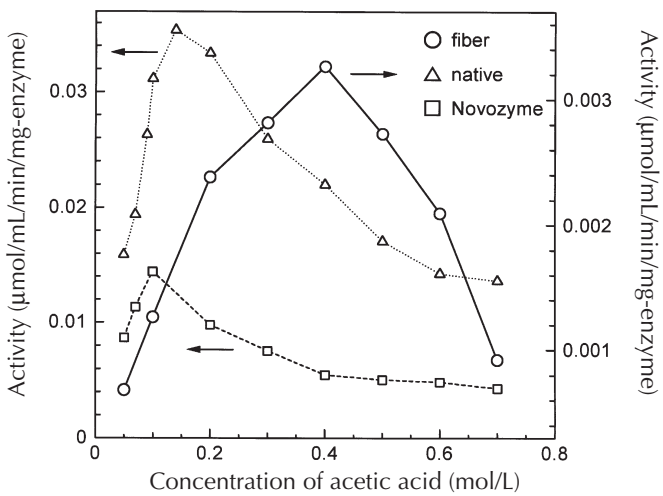

FIG. 4. Effect of concentration of acetic acid on lipase-catalyzed synthesis of geranyl acetate at 30°C. Geraniol was kept at a concentration of 0.2 mol/L, and acetic acid concentration was changed. See Figure 2 for company source.

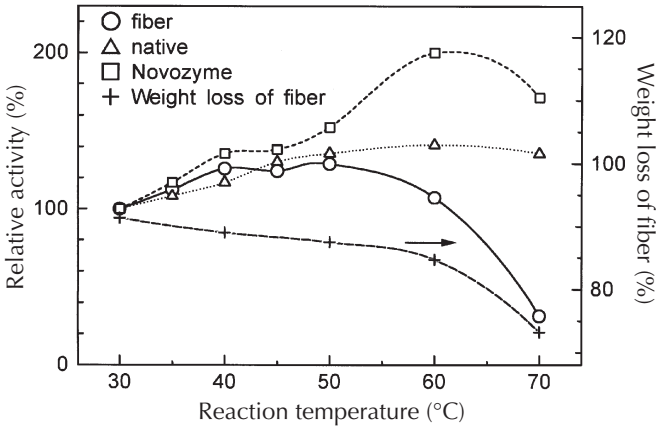

FIG. 5. Temperature profile of lipase-catalyzed esterification of geraniol with acetic acid and weight loss of the gel fiber after 3-h incubation in heptane. Esterification was carried out for 5 h. The activity at 30°C was taken as 100%. Weight loss of fiber (%) = [(weight of fiber after incubation)/(weight of fiber before incubation)] × 100. See Figure 2 for company source.

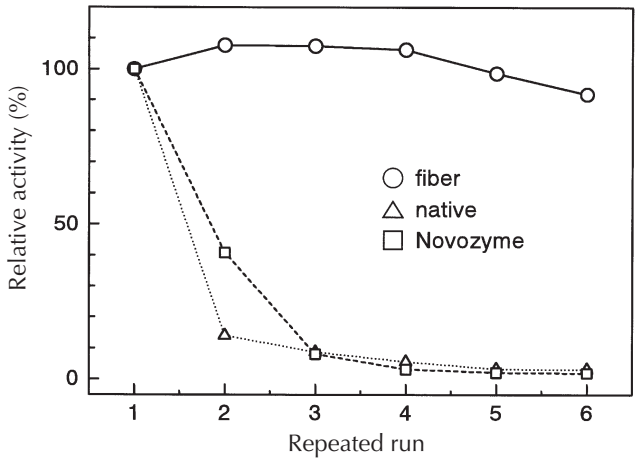

FIG. 6. Effect of repeated runs on lipase-catalyzed esterification of geraniol with acetic acid at 30°C. Activity of the first run was taken as 100%. One reaction period was 20 h. See Figure 2 for company source.

of the reaction temperature on the esterification and the weight loss of the fiber after standing in heptane at a given temperature for 3 h. All the esterification reactions were performed for 5 h, and the activity at 30°C was taken as 100%. The native lipase showed resistance to high temperature in the hydrophobic organic solvent system employed here and retained stable activities across the temperature range of 30 to 70°C. Novozyme showed an increase of activity with increasing temperature, and maximum activity at 60°C. In spite of the thermal resistance of native lipase, the activity of fiber-immobilized lipase was steeply depressed at higher temperature. In addition, as shown in Figure 5, a considerable weight loss of gel fiber was observed, especially at higher temperature. It was considered that the weight loss approximately corresponded to water loss from the fiber because only water would be removed from the gel during the incubation in heptane. As mentioned above, the water content of this fiber-immobilized lipase constituted about 50% of the fiber weight. This dehydration occurred simultaneously with the gel-fiber shrinkage caused by thermal cellulose crystallization. Since the gel network was tightened owing to both thermal contraction and dehydration, the number of effective active sites available for the reaction decreased. It was therefore concluded that the substrate could not easily contact the active site of the lipase entrap-immobilized in the gel fiber, and that this led to decreasing activity.

The effects of temperature and substrate concentration on the activity of esterification of citronellol with acetic acid were also investigated, though not systematically, and the results were roughly similar to those of geranyl acetate synthesis.

In Figure 6, the activities of native, Novozyme, and fiber-immobilized lipase are plotted against the number of reaction cycles. One reaction period was 20 h, and lipases were washed with heptane after each run. After six runs, the fiber-immobilized lipase retained more than 90% of the activity level of the first run, whereas the activity levels of native lipase and Novozyme dropped steeply after two experiments. The decrease of native lipase activity was caused by the decreasing contact area between enzyme and substrate, which resulted from hydration and aggregation of the enzyme by the water produced during the reaction. Indeed, the native lipase was not suitable for reuse, because it formed a gelatinous mass by hydration. In the case of Novozyme, the drop in activity was probably due to the produced water being adsorbed on the macroporous acrylic resin, thereby preventing esterification. Moreover, enzymes may have escaped from the support during the reaction or by washing.

We examined the leakage of lipase from Novozyme and fiber-immobilized lipase into an aqueous solution by monitoring the changes in the absorption of the solution at 280 nm. The results are shown in Figure 7. Because the acetone used for producing the gel fiber might affect the measurement of absorbance at 280 nm, a solution with gel fiber containing no lipase (blank) was also examined. An increase in absorbance was observed in Novozyme solution during 24-h incubation,

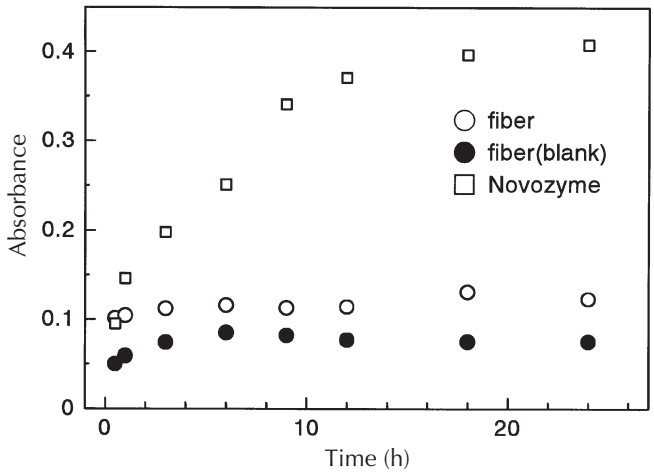

FIG. 7. Time course of the absorbance at 280 nm of the aqueous solution containing immobilized lipase. Solutions were shaken at 30°C. See Figure 2 for company source.

demonstrating that lipase was desorbed from the support. In the case of fiber-immobilized lipase solution, only slight absorbance was seen in the initial stage, and this absorbance did not increase. Although small amounts of lipase loosely immobilized near the surface of the gel fiber escaped at the beginning of incubation, there was almost no leakage of lipase after that. It indicated that lipase, which is a globular protein of 33273 daltons (17), was entrap-immobilized rigidly in the gel fiber. Furthermore, since fiber-immobilized lipase can be more easily recovered from solution than powdered native lipase or fine bead-shaped Novozyme, our entrap-immobilization method is highly advantageous for practical applications.

Lipase from *C. antarctica* was entrap-immobilized in cellulose acetate-TiO_2 gel fiber. This fiber-immobilized lipase appeared to be an effective catalyst for terpene ester synthesis. In addition to its high reusability and ease of handling, the activity of the fiber-immobilized lipase was not affected by the bulk water content or the produced water. Since the gel fiber is stable in both organic and aqueous solutions, our entrap-immobilization method could be applied to the other enzyme immobilizations for various reaction systems. However, further investigation will be needed into improvements of activity depression by immobilization, e.g., thinning of fibers (increasing fiber surface area) and co-doping of the surface-active reagent (softening gel).

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