Synthesis of (*Z*)-3-Hexen-1-yl Acetate by Lipase Immobilized in Polyvinyl Alcohol Nanofibers

Koji Nakane, Toshiki Hotta, Takashi Ogihara, Nobuo Ogata, Shinji Yamaguchi

Materials Science and Engineering Course, Graduate School of Engineering, University of Fukui, Fukui 910-8507, Japan

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ABSTRACT: Polyvinyl alcohol (PVA)-nanofibers-immobilized lipase were formed by electrospinning. The specific surface area of the nanofiber (5.96 m^2/g) was about 250 times larger than that of PVA-film-immobilized lipase (0.024 m^2/g). The PVA-nanofibers-immobilized lipase were used as the catalyst for the esterification of (*Z*)-3-hexen-1-ol (leaf alcohol) with acetic acid in hexane. The activity of the nanofiber is equivalent to that of commercially available immobilized lipase (Novozym-435). The ester conversions of the nanofibers, Novozym-435, the film and lipase powder reached 99.5% at 5 h, 100% at 5 h, 11.5% at 6 h, and 81.1% at 5.75 h, respectively. The nanofibers-immobilized lipase showed higher activity for the esterification than the film-immobilized lipase and lipase powder, probably because it has high specific surface area and high dispersion state of lipase molecules in PVA matrix. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 106: 863–867, 2007

Key words: polyvinyl alcohol; lipase; nanofiber; electrospinning; immobilization

INTRODUCTION

Recently, much attention has been paid to the polymeric nanofibers formed by electrospinning. Electrospinning is a process by which nanofibers several hundred nanometers in diameter are easily produced.[1–4] The average diameter of the fibers produced by this method is at least one or two orders of magnitude smaller than those by conventional fiber production methods like melt or solution spinning.[5] As a result, the electrospun fibers have a high specific surface area.[4] These nanofibers are well-suited to be used as chemical reaction fields.

Water-soluble polymers such as polyvinyl alcohol (PVA) are considered to be useful materials for creating a high-dispersion state of enzymes in polymer supports. In this study, we utilized polymeric nanofibers as an enzymatic reaction field. Namely, PVA-nanofibers-immobilized lipase (EC 3.1.1.3) formed by electrospinning was used as a catalyst for flavor ester synthesis in organic media.

The use of lipase as a catalyst of esterification in an organic solvent has been well examined.[6] For use in esterification, immobilized lipase has certain advantages over lipase powder, e.g., the reusability of the enzyme, prevention of solvent toxicity and a reduction of the influence of product water. Numerous supports for the immobilization of lipases have been investigated.[7–12] In most cases, supports such as celite (a sort of clay mineral) and anion-exchanger resin have been selected for adsorption. But desorption of the enzyme occurs by washing. Furthermore, covalent attachment and crosslinking on supports often cause enzyme denaturation. For industrial application, adsorption, and entrapment are often used because the process costs are relatively low and the process can be carried out under mild conditions.

We previously reported the synthesis of terpene ester (citronellyl acetate) using PVA-nanofibers-immobilized lipase.[13] The nanofibers showed higher activity and stability for the ester synthesis than commercially available immobilized lipase. However, we did not examine the effect of the specific surface area of the nanofibers upon the lipase activity or the effect of water (a byproduct of esterification) upon the lipase activity. This paper deals with the esterification of (*Z*)-3-hexen-1-ol, which is chain aliphatic alcohol, with acetic acid in a hexane solution using PVA-nanofibers-immobilized lipase (Scheme 1). The effects of the surface area of the nanofibers and the product water upon the lipase activity were investigated in this study. Esters such as (*Z*)-3-hexen-1-yl acetate are important flavor and fragrance compounds (green note compounds) used in the food and cosmetic industries.[14,15] Industrial production of flavor esters is accomplished via a nonspecific chemical process requiring complicated purification steps. Lipase-catalyzed esterification can be performed

Correspondence to: K. Nakane (nakane@matse.fukui-u.ac.jp).

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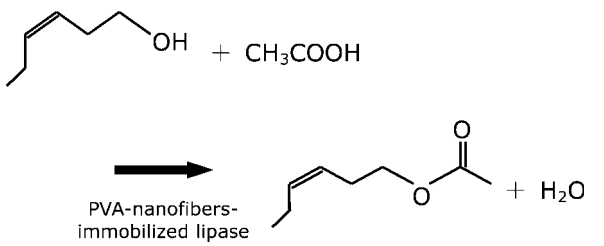

Scheme 1 Synthesis of (*Z*)-3-hexen-1-yl acetate using PVA-nanofibers-immobilized lipase.

under more moderate conditions and yields a high-quality product.

There are some reports about the usage of erectrospun nanofibers as an enzyme-immobilization support (carrier-binding method) in water system.[16–20] But the usage of nanofibers as an enzyme-immobilization support (entrapping method) in organic media has not been seen except for our previous report.[13]

EXPERIMENTAL

Materials

All reagents were of commercially available reagent grade. PVA (degree of polymerization: 1,500) was obtained from Wako Pure Chemicals Industries (Japan). Lipase powder (Chirazym L-2, Lyo., lipase B from Candida Antarctica) was purchased from Roche Diagnostics, Switzerland. Novozym-435 (lipase B from Candida Antarctica immobilized on macroporous polyacrylate resin: bead size 0.3–0.9 mm, activity ~ 10,000 propyl laurate units per gram) was procured as a gift sample from Novozymes Japan.

Formation of PVA-nanofibers-immobilized lipase by electrospinning

Figure 1 illustrates the electrospinning apparatus. The 10 wt % PVA aqueous solution in which lipase (5 wt %/g-PVA) was prepared (spinning solution) was loaded into a plastic syringe equipped with a needle. The spinning solution was extruded by a syringe pump, and the extrusion rate was 0.015 mL/min. A voltage of 25 kV was applied to the needle, and the electrically charged spinning solution was jetted out from the needle tip (polymer jet). The discharged polymer jet undergoes instability and an elongation process. Meanwhile, the solvent (water) evaporates, leaving behind the charged polymeric nanofibers. Then, PVA-nanofibers-immobilized lipase were deposited on a collector (copper plate) as a non-woven mat. The collector was grounded. The distance between the tip of the needle and the collector was 10 cm.

The morphology of PVA-nanofibers-immobilized lipase was observed by a Hitachi S-2300 scanning electron microscope (SEM) (Japan).

The nitrogen adsorption isotherms (−196°C) of the nanofibers were measured by Micromeritics TriStar 3000 (USA).

PVA-film-immobilized lipase was formed by the solution casting technique (PVA/lipase mixed aqueous solution). The solvent (water) was dried at room temperature. The surface area of the PVA film could not be estimated by the nitrogen adsorption method because the film is a nonporous material (low surface area). Thus, the surface area of the film was calculated from its length and thickness.

Esterification method

Esterification was carried out in screw-capped glass tubes. Lipase powder (0.003 g), Novozym-435 (0.06 g), or PVA-nanofibers (or film)-immobilized lipase (0.06 g) were added to a 20-mL hexane solution containing 0.2mol/L (*Z*)-3-hexen-1-ol (leaf alcohol) ($C_6H_{12}O$) and 0.1 mol/L acetic acid, respectively. The amount of enzymes in the PVA nanofibers and film was the same as that in the lipase powder in the reaction solutions. Linalool (0.2 mL) was also added as an internal standard. The solutions were shaken at 80 strokes/min in a thermostatic water bath at 30°C. An aliquot was periodically withdrawn from the reaction solution and analyzed by gas chromatography Shimadzu GC-12A (Japan) (flame-ionization detector, N_2 carrier gas) packed polyethylene glycol (PEG) 20*M* stainless column (3 m); column temperature 100°C; injection and detection temperature 230°C. The lipase activities were evaluated from the peak area of the chromatogram and the working curve prepared.

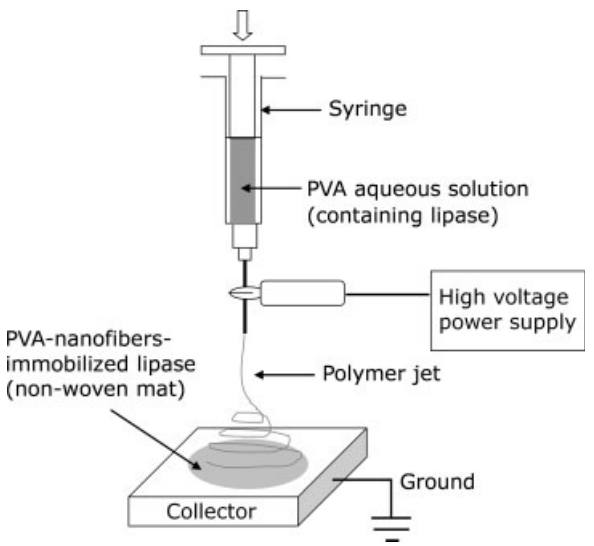

Figure 1 Schematic illustration of electrospinning apparatus.

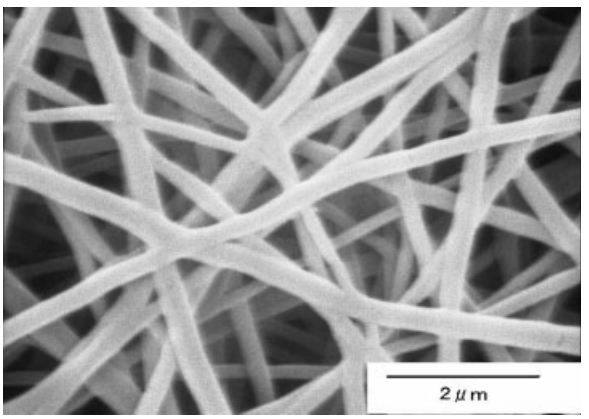

Figure 2 SEM image of PVA-nanofibers-immobilized lipase.

The effect of the reaction cycle on activity was examined for each lipase. One reaction period was 3 h. After each run, the lipase preparation was washed 2 times with fresh hexane (20 mL). A reaction temperature profile was taken for PVA-nanofibers-immobilized lipase, Novozym-435 and lipase powder. In this case, heptane (b.p. 98.4°C) was used as the solvent.

RESULTS AND DISCUSSION

Formation of PVA-nanofibers-immobilized lipase

Figure 2 shows photograph of the PVA-nanofibers-immobilized lipase formed by electrospinning. The lipase is physically entrap-immobilized among the PVA chains and distributed throughout the nanofibers. A non-woven mat of nanofibers was used as the catalyst for esterification.

Figure 3 shows the nitrogen adsorption isotherms (−196°C) of nanofibers and lipase powder. The adsorption amounts of pure PVA nanofibers are slightly higher than those of PVA-nanofibers-immobilized lipase. The amount of adsorption increases in the high-relative-pressure range above 0.9, and is due to the condensation of nitrogen at contact points between the fibers. The specific surface areas of nanofibers were calculated by a BET equation from these isotherms. The specific surface area of pure PVA nanofibers (8.12 m^2/g) was higher than that of PVA-nanofibers-immobilized lipase (5.96 m^2/g). The diameter of the PVA-nanofibers-immobilized lipase might be slightly larger than that of the pure PVA fibers. This would be due to the difference in the viscosities of the spinning solutions.[13] The specific surface area of lipase powder obtained from the isotherm was 1.52 m^2/g.

PVA nanofibers swell and dissolve in water. Consequently, the fibers cannot be used as an enzyme-immobilization support in an aqueous medium.

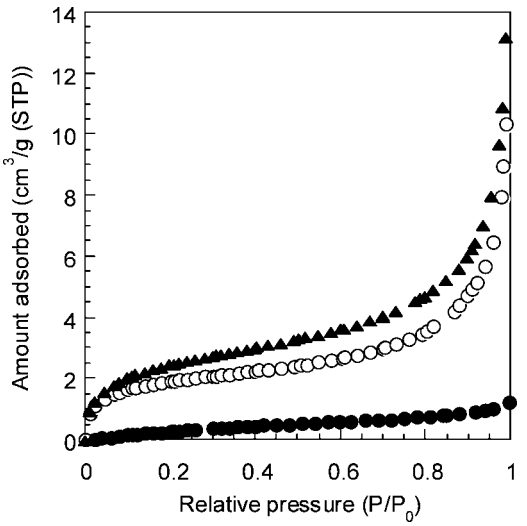

Figure 3 Nitrogen adsorption isotherms (−196°C) of PVA-nanofibers-immobilized lipase (○), lipase powder (●) and pure PVA nanofibers (▲).

Thus, we attempted to use them as the catalyst for esterification in a non-aqueous medium.

Ester synthesis using PVA-nanofibers-immobilized lipase

Figure 4 shows the time courses of the synthesis of (*Z*)-3-hexen-1-yl acetate. The ester conversion of PVA-nanofibers-immobilized lipase increased with increasing reaction time and reached 99.5% after 5 h. There was no activity for the pure PVA nanofibers. The esterification rate for PVA-nanofibers-immobilized lipase was equivalent to that for Novozym-435.

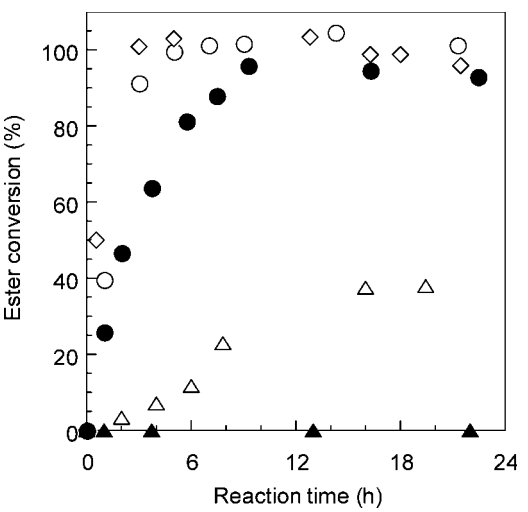

Figure 4 Effect of reaction time on lipase-catalyzed synthesis of (*Z*)-3-hexen-1-yl acetate at 30°C. [○: PVA-nanofibers-immobilized lipase, ●: lipase powder, △: PVA-film-immobilized lipase, ▲: PVA nanofibers (no lipase), ◇: Novozym-435].

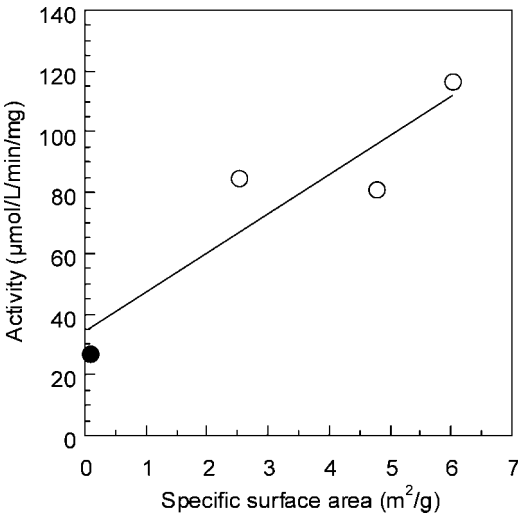

Figure 5 Effect of specific surface area of PVA-immobilized lipase on activity. One reaction period was 3h. [○: PVA nanofibers, ●: PVA film].

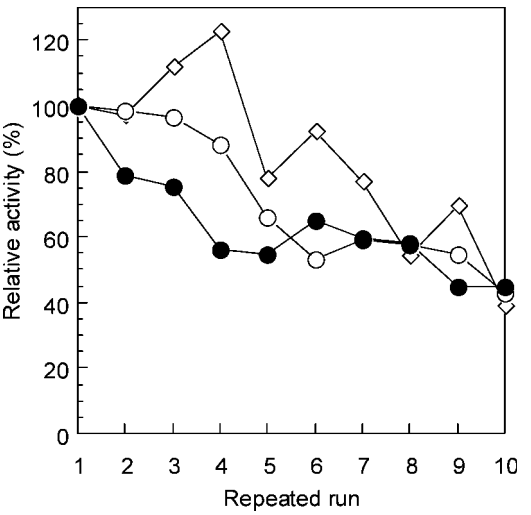

Figure 6 Effect of repeated runs on lipase-catalyzed synthesis of (*Z*)-3-hexen-1-yl acetate at 30°C. Activity of the first run was taken as 100%. One reaction period was 3h. [○: PVA-nanofibers-immobilized lipase, ●: lipase powder, ◇: Novozym-435].

And the rate for the nanofibers was faster than that for lipase powder. This is due to the difference in dispersion states of each lipase.[13] The lipase powder is a crude purification product; thus, lipase molecules are engulfed by impurities (proteins) that are insoluble components in organic solvents. On the other hand, the dispersion state of lipase molecules in PVA nanofibers is relatively good because the lipase powder is dissolved in the PVA aqueous solution before the electrospinning. Also, the specific surface area of lipase powder is lower than that of nanofibers as noted earlier.

The esterification rate for PVA-film-immobilized lipase is very slow (Fig. 4), although the lipase is dispersed homogeneously in PVA film as with PVA nanofibers. The major difference between the nanofibers and the film is their specific surface areas. The specific surface area of the PVA-nanofibers-immobilized lipase (5.96 m^2/g) is about 250 times larger than that of PVA-film-immobilized lipase (0.024 m^2/g).

Figure 5 shows the relationship between the specific surface areas and the activities for the PVA-nanofibers-immobilized lipase (white circle) and the PVA-film-immobilized lipase (black circle). The diameter (the specific surface area) of the PVA nanofibers was controlled by changing the electrospinning conditions, the concentration of the PVA solution (10, 15 wt %) and the applied voltage (10, 17, 25 kV). As seen in the figure, there is a linear relationship between the surface area and the activity. This suggests that the enzyme reaction occurs in the neighborhood of the nanofiber surface.

In Figure 6, the activities of lipase powder, Novozym-435 and PVA-nanofibers-immobilized lipase are plotted against the number of reaction cycles. The activity of the first run was taken as 100%. After 10 runs, the PVA-nanofibers-immobilized lipase retain about 40% of the activity level of the first run. The same tendencies were observed for lipase powder and Novozym-435. The decrease of the relative activity is mainly due to the product water being adsorbed onto the lipase molecules, thereby preventing esterification.[21] Indeed, lipase powder is not suitable for reuse, because it forms a gelatinous mass by hydration. Novozym-435 is also hard to recover because it is a powder. On the other hand, a nonwoven mat of nanofibers can be recovered very easily from the reaction solution.

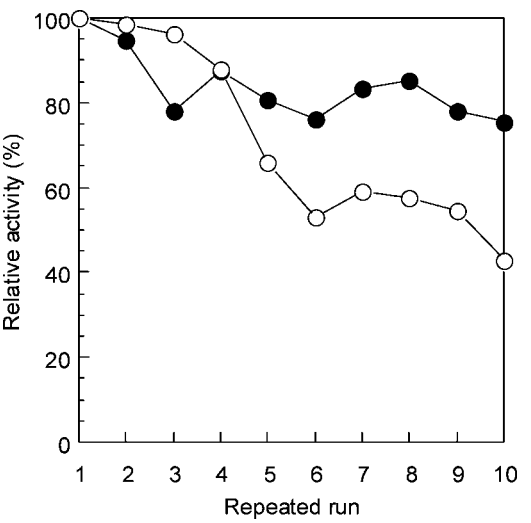

Figure 7 Effect of repeated runs on lipase-catalyzed synthesis of (*Z*)-3-hexen-1-yl acetate. [○: PVA-nanofibers-immobilized lipase (non-dried), ●: PVA-nanofibers-immobilized lipase (dried each time)].

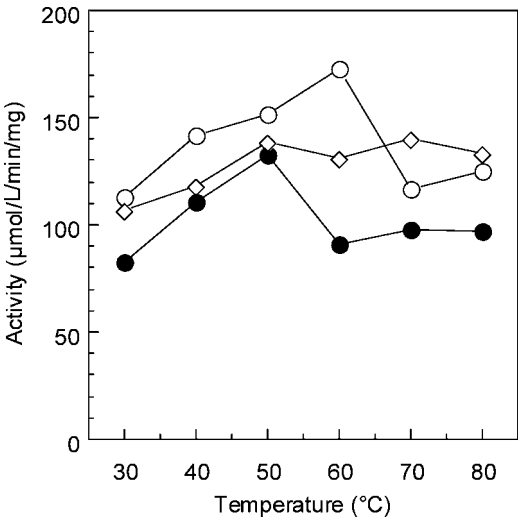

Figure 8 Effect of reaction temperature on lipase-catalyzed synthesis of (*Z*)-3-hexen-1-yl acetate. Esterification was carried out in heptane for 3 h. [○: PVA-nanofibers-immobilized lipase, ●: lipase powder, ◇: Novozym-435].

To investigate the inhibition of the product water on the esterification, PVA nanofibers used in esterification were dried *in vacuo* at room temperature for 20 h with each reaction cycle. Figure 7 shows the effect of repeated runs on the relative activity of the PVA-nanofibers-immobilized lipase. The data of non-dried nanofibers is same as that in Figure 6. The nanofibers dried each time maintained a higher activity level (75.4% at 10 runs) than the non-dried nanofibers (42.7% at 10 runs). Thus, the activity level of reused nanofibers can be improved by the removal of excess water.

In general, the thermal stability of enzymes can be expected to improve by entrap-immobilization. Figure 8 shows the effect of the reaction temperature on esterification. As the Figure demonstrates, PVA-nanofibers-immobilized lipase are more active in a higher temperature range because of the prevention of the thermal denaturation of lipase molecules by entrap-immobilization.

CONCLUSIONS

PVA-nanofibers-immobilized lipase formed by electrospinning were used as the catalyst for the esterification of (*Z*)-3-hexen-1-ol with acetic acid in hexane. The immobilization procedure was very simple and practical. This nanofiber showed higher activity for esterification than lipase powder and PVA-film-immobilized lipase. This is due to the high specific surface area of the nanofibers and the high dispersion state of lipase molecules in the PVA matrix. The activity of the nanofibers became higher with increased specific surface area, indicating that esterification occurs in the neighborhood of the nanofiber surface. Also, the activity of the nanofibers was equivalent to that of Novozym-435. Compared to Novozym-435 and lipase powder, the non-woven mat of nanofibers can be recovered far more easily from the reaction solution. Thus, we conclude that PVA nanofibers are a very useful enzyme support for ester synthesis in organic media.

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