Esterification of Lauric Acid Using Lipase Immobilized in the Micropores of a Hollow-Fiber Membrane

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ABSTRACT: A porous anion-exchange hollow-fiber membrane was prepared by radiation-induced graft polymerization and chemical modification to immobilize lipase for enzymatic reaction in an organic solvent. The amount of anion-exchange group introduced to the porous hollow-fiber membrane was 2.5 mol/kg_{fiber}. A lipase solution was allowed to permeate through the porous anion-exchange hollow-fiber membrane, and lipase molecules that adsorbed onto the grafted polymer brush were cross-linked with glutaraldehyde. The lipase was immobilized at a density of 0.14 kg_{lipase}/kg_{fiber}, which was equivalent to a degree of multilayer binding of 20. Esterification was carried out by passing a solution of lauric acid and benzyl alcohol in anhydrous isooctane through the lipase-immobilized membrane, and lipase activity was determined. A reaction percentage of 50% was achieved at space velocity 68 h^{-1}. The maximum immobilized lipase and native lipase activities were 8.9 and 0.38 mol/(h·kg_{lipase}), respectively. Thus, the activity of the immobilized lipase was 23.4 times higher than that of the native lipase.

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KEY WORDS: Enzymatic esterification, graft polymerization, hollow-fiber, immobilization, lauric acid, lipase, polymer brush, *Rhizopus* sp.

Lipases are widely used for biotechnology applications in the dairy industry, in oil processing, and for the preparation of enantiomerically pure pharmaceuticals (1). Biodiesel fuels are expected to be substitutes for conventional fossil fuels and have been industrially produced from vegetable oils in North America and Europe (2,3). Lipases have been used to make the methyl esters or ethyl esters used in biodiesel. These fuels have the environmental advantages of biodegradability, renewability, and improved exhaust emissions (4,5). Lipases used in organic solvents can benefit from immobilization, because they have low solubility in organic solvents or in an anhydrous environment.

Enzymes can be modified for use in organic media by being coated with surfactant (6,7). The hydrophilic group of the surfactant is directed toward the enzyme, and the hydrophobic group is directed toward the organic medium. Surfactant-coated enzymes are considered to be activated because of their increased affinity for lipophilic substrates and protection from inactivation by organic solvents. We previously prepared surfactant-coated lipases from *Pseudomonas* sp. using various kinds of surfactants (8). Surfactant-coated lipases having a long, branched hydrophobic group as the surfactant tail and six hydroxyl groups as the surfactant head (glutamic acid dioleyl ester ribitol) was highly activated in organic media.

In applications, lipases are immobilized on a support to obtain the advantages of easy recovery of the enzyme from products, continuous operation, and increased enzyme stability. Conventional methods of enzyme immobilization include the entrapment of enzymes in gel matrices, such as carrageenan and agarose (9), and immobilization on membranes (10).

A novel method of immobilizing enzymes onto porous hollow-fiber membranes modified using radiation-induced graft polymerization has been suggested (Fig. 1). Support for the enzyme is achieved by using a porous hollow-fiber membrane with a grafted polymer brush. An ion-exchange group containing the grafted polymer brush on the porous hollow-fiber membrane extends from the pore surface toward the pore interior because of mutual repulsion, and captures the enzyme *via* electrostatic interactions. Permeation of the substrate solution through the membrane minimizes diffusional mass-transfer resistance of the substrate to the immobilized enzyme, and hence enhances the overall enzymatic reaction. Aminoacylase (11), cycloisomaltooligosaccharide glucanotransferase (12), and urease (13) have been immobilized in this manner, and they exhibit higher enzymatic activity than conventional immobilized enzymes in aqueous solution.

To our knowledge, immobilization of enzymes attached to an ion-exchange group containing a polymer brush grafted onto a porous hollow-fiber membrane for applications in organic media has not been reported. When an immobilized enzyme on an ion-exchange group containing a polymer brush grafted onto a porous hollow-fiber membrane is used for an esterification reaction in organic media, the activity of the immobilized en-

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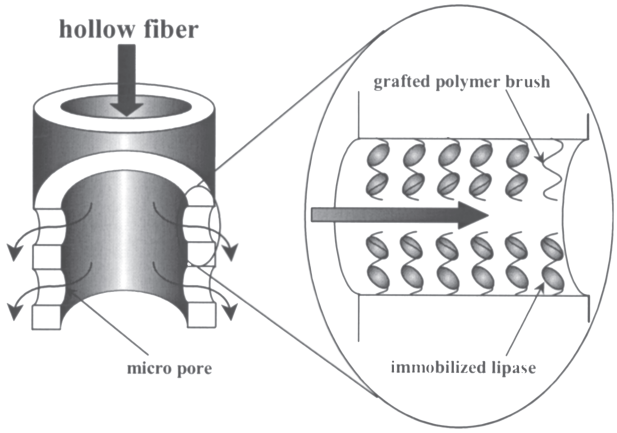

FIG. 1. Schematic illustration of lipase immobilized onto the pores of a porous hollow-fiber membrane: porous hollow-fiber membrane; enzyme immobilized onto a polymer brush grafted on the pore surface.

zyme in the organic media is predicted to increase, because the grafted polymer chain will act as a hydrophobic surfactant.

The objectives of this study were twofold: (i) to immobilize lipase originating from a *Rhizopus* sp. onto a porous hollow-fiber membrane using an ion-exchange group containing a polymer brush; and (ii) to evaluate the activity of lipase immobilized on the porous hollow-fiber membrane during permeation of a substrate solution dissolved in organic media. The polymer brush could function as a lipase-coated layer and as a lipase-immobilization support to produce a high rate of enzymatic reaction.

MATERIALS AND METHODS

Materials. A porous hollow-fiber polyethylene membrane with inner and outer diameters of 1.9 and 3.1 mm, respectively, a pore size of 360 nm, and porosity of 71% was used as a trunk polymer for grafting. This membrane was supplied by Asahi Kasei Corporation (Tokyo, Japan). Glycidyl methacrylate (GMA) was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan) and used without further purification. Lipase D from *Rhizopus oryzae* was a gift from Amano Enzyme Inc. (Nagoya, Japan). Other reagents were of analytical grade or higher.

Preparation of the porous anion-exchange hollow-fiber membrane. The scheme for preparing the porous anion-exchange hollow-fiber membrane is shown in Figure 2. The membrane was irradiated at 200 kGy to form radicals by an electron beam in a nitrogen atmosphere at ambient temperature using a cascade-type accelerator (Dynamitron model IEA 3000-25-2; Radiation Dynamics Inc., Edgewood, NY). The irradiated membrane was then immersed in 10% (vol/vol) GMA/ethanol solution at 40°C for 12 min. The degree of GMA grafting (dg) was defined as:

$$\text{dg}\ (\%) = \frac{\text{mass of polymer} - \text{GMA grafted}}{\text{mass of trunk polymer}} \times 100 \qquad [1]$$

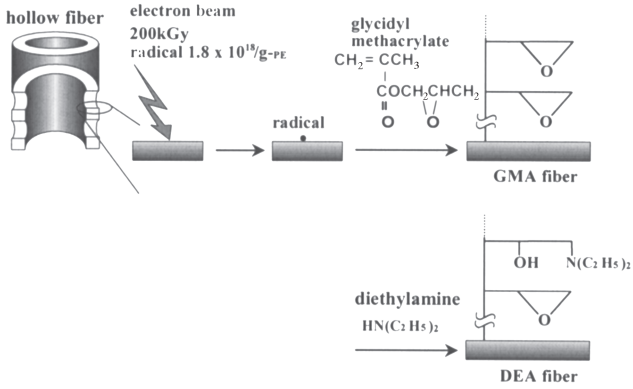

FIG. 2. Introduction of a diethylamino group into a grafted polymer brush. GMA, glycidyl methacrylate; DEA, diethylamine.

The resulting membrane was referred to as GMA fiber.

To introduce an anion-exchange group to the grafted polymer chains, GMA fiber was reacted with 50% (vol/vol) diethylamine (DEA) solution for 1.5 h at 40°C. Molar conversion of an epoxy group in the grafted polymer brush to an anion-exchange group was defined as:

$$\text{molar conversion}\ (\%) = \frac{\text{moles of diethylamino group after functionalization}}{\text{moles of epoxy group before functionalization}} \times 100 \qquad [2]$$

where the moles were calculated from fiber mass change after reaction (functionalization or grafting). The resulting membrane was referred to as DEA fiber.

Pure water permeability of the membrane was determined in the dead-end mode and expressed as flux at a constant transmembrane pressure of 0.1 MPa at 25°C:

$$\text{pure water flux (m/h)} = \frac{\text{permeation rate (m}^3\text{/h)}}{\text{inside surface area of the porous hollow fiber (m}^2\text{)}} \qquad [3]$$

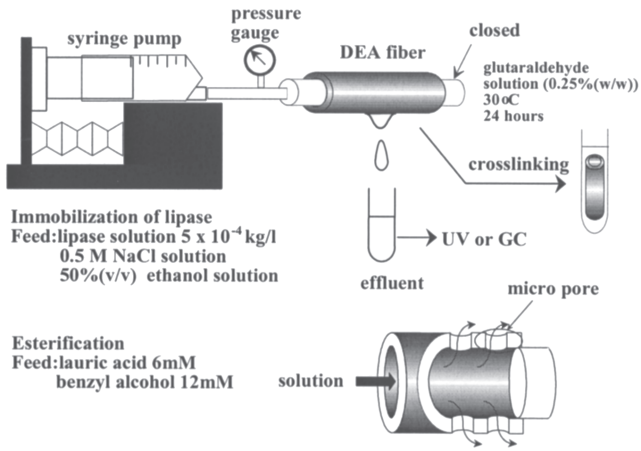

FIG. 3. Experimental apparatus for lipase immobilization onto DEA fiber, and the esterification reaction. For abbreviation see Figure 2.

Immobilization of lipase on the pores of the hollow fiber. A DEA fiber (effective length: 2 cm) was placed onto an experimental apparatus as shown in Figure 3. Lipase solution (5×10^{-4} kg/L) in 10 mM phosphate buffer (pH 7.5) was forced to permeate from the inside to the outside of the membrane at a permeation rate of 30 mL/h at ambient temperature. The concentration of lipase in the effluent penetrating the outside surface of the DEA fiber was determined from UV absorption at 280 nm.

The amount of lipase adsorbed on the DEA fiber was calculated as follows:

$$\text{amount of lipase adsorbed (kg/kg}_{\text{fiber}}) = \int_0^v \frac{(C_0 - C)}{W} dv \qquad [4]$$

where C_0 and C are the concentrations in the feed and the effluent (kg/L), respectively. v and W are the effluent volume (L) and membrane mass of DEA fiber in the dry state (kg), respectively.

The lipase-adsorbed membrane was immersed in 0.25% (vol/vol) glutaraldehyde solution dissolved in 10 mM phosphate buffer (pH 7.5) at 30°C for 24 h to covalently cross-link lipase molecules. Subsequently, uncross-linked lipase was eluted by permeation with 0.5 M NaCl solution and 50% (vol/vol) ethanol solution. The amounts of lipase immobilized on the membrane and the percentage of cross-linking were defined as:

$$\text{amount of lipase immobilized (kg/kg}_{\text{fiber}}) = A_a - A_e \qquad [5]$$

$$\text{cross-linking percentage (\%)} = \frac{\text{amount of lipase immobilized}}{\text{amount of lipase adsorbed}} \times 100 \qquad [6]$$

where A_a is the amount of lipase adsorbed on the membrane and A_e is the amount of lipase eluted from the membrane.

The resulting lipase-immobilized porous hollow-fiber membrane was referred to as lipase fiber.

Esterification reaction using immobilized lipase. A prepared lipase fiber was placed on the permeation apparatus shown in Figure 3, and one end was blocked. A substrate solution was prepared by dissolving lauric acid and benzyl alcohol in anhydrous isooctane. Concentrations of lauric acid and benzyl alcohol were 6 and 12 mM, respectively. The substrate solution was fed into the lipase fiber using a syringe pump and allowed to permeate through the micropores of the lipase fiber. Based on the membrane volume including micropores, the space velocity (SV) of the substrate solution was defined as follows:

$$\text{SV (h}^{-1}) = \frac{\text{flow rate of substrate solution}}{\text{membrane volume excluding the lumen part}} \qquad [7]$$

The SV of the substrate solution varied from 61 to 420 h^{-1}. Esterification was carried out in a temperature-controlled room at 25°C.

In control experiments, batch reactions were carried out using lipase fiber or native lipase. Reactions were carried out in a 50-mL screw-capped vessel with an agitation speed of 200 rpm. The reaction was initiated by adding native lipase, lipase fiber, or lipase fiber without glutaraldehyde treatment (cross-linking) to 10 mL of substrate solution.

Analysis. The concentration of lauric benzyl ester as a product in the effluent was determined with a gas chromatograph (Hewlett-Packard 5890) equipped with an FID and a 15-m capillary column (DB-1; Agilent Technologies Inc., Palo Alto, CA). The flow rates of He (carrier gas), air, and H_2 were 16, 360, and 30 mL/min, respectively. Temperatures of the injector and FID were 70 and 350°C, respectively. The column temperature was increased from 70 to 200°C at 20°C/min. The reaction percentage and immobilized enzyme activity were defined as follows:

$$\text{reaction percentage (\%)} = \frac{\text{concentration of product}}{\text{initial concentration of substrate}} \times 100 \qquad [8]$$

$$\text{immobilized enzyme activity (mol/(h}\cdot\text{kg}_{\text{lipase}})) = \frac{(C_p \times F)}{\text{amount of immobilized enzyme}} \qquad [9]$$

where C_p was the concentration of the product (lauric benzyl ester), and F was the flow rate of the substrate solution. In the batch reactions, lipase activity was defined as the initial formation rate of benzyl laurate per kilogram of lipase [mol/(h·kg-$_{\text{lipase}}$)].

RESULTS AND DISCUSSION

Properties of the anion-exchange hollow-fiber membrane. The density of the diethylamino group of the porous hollow-fiber membrane, prepared at a GMA grafting of 160% and 65% molar conversion of the epoxy group in the grafted polymer

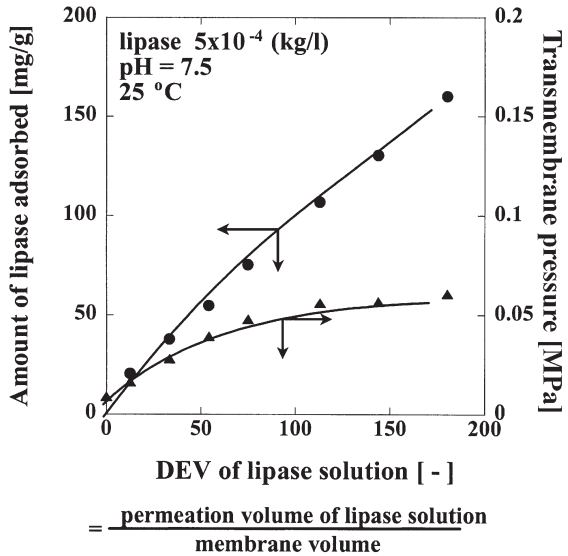

FIG. 4. Effects of dimensionless effluent volume (DEV) on the amount of lipase adsorbed and transmembrane pressure.

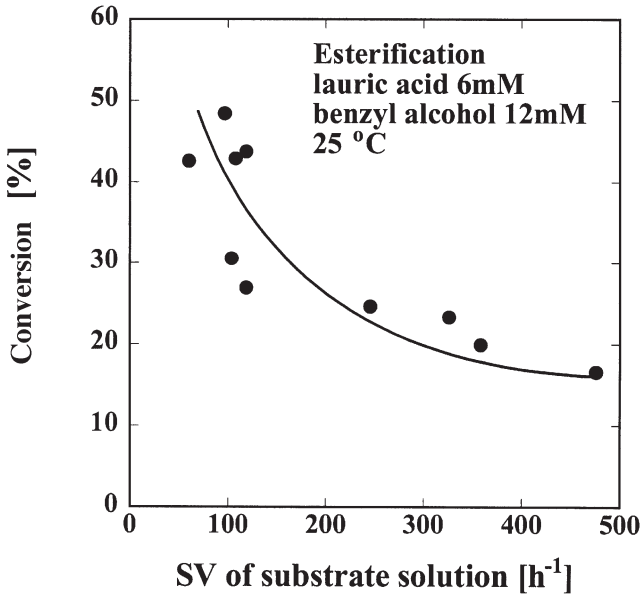

FIG. 5. Relationship between the reaction percentage and the space velocity (SV) of a substrate solution using lipase immobilized onto DEA fiber. The reaction was carried out at 25°C, with the concentrations of lauric acid and benzyl alcohol as substrates being 6 and 12 mM, respectively. For other abbreviation see Figure 2.

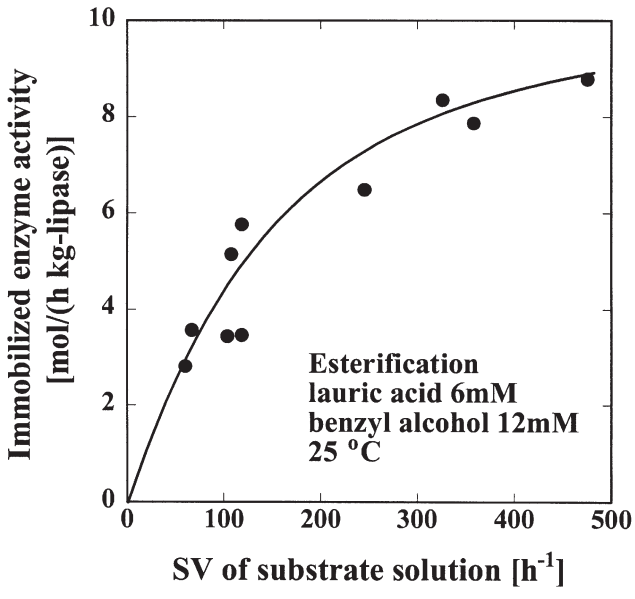

FIG. 6. Effects of SV on immobilized enzyme activity. The reaction was carried out at 25°C, with concentrations of lauric acid and benzyl alcohol as substrates being 6 and 12 mM, respectively. For abbreviation see Figure 5.

brush, was 2.5 mol/kg_{fiber}. GMA grafting onto the porous hollow-fiber membrane and subsequent chemical modification induced swelling of the membrane, i.e., the inner and outer diameters of the DEA fiber were 2.4 and 4.1 mm, respectively, compared with the corresponding diameters of 1.9 and 3.1 mm of the trunk porous hollow-fiber membrane.

Pure water fluxes of trunk polymer, GMA fiber, and DEA fiber were 2.5, 1.8, and 0.5 m/h, respectively. The pore diameter of the GMA fiber decreased when the polymer brush was grafted into the pores. This is believed to indicate that the anion-exchange grafted polymer brush of the DEA fiber was extended because of its mutual electrostatic repulsion, decreasing the pore diameter of the DEA fiber.

Amount of lipase immobilized onto the hollow fiber. The amount of lipase adsorbed onto the DEA fiber as a function of the permeation volume of the lipase solution is shown in Figure 4 together with the transmembrane pressure of the lipase-adsorbed membrane. The abscissa represents the dimensionless effluent volume (DEV), defined as the ratio of effluent volume to membrane volume, excluding the lumen. The amount of lipase adsorbed increased linearly with increases in the permeation volume of the lipase solution. The amount of adsorbed lipase was 0.160 kg/kg_{fiber} at a DEV of 190. Transmembrane pressure increased during permeation of the lipase solution. The anion-exchange group containing a grafted polymer brush is known to extend into its pore interiors after adsorption of the lipase (14).

Immobilization of the lipase could have resulted from ionic, covalent, or nonspecific interactions. Following cross-linking of the lipase with glutaraldehyde, 0.5 M NaCl and a 50% (vol/vol) ethanol solution were passed through the fiber and 13% of the adsorbed lipase was eluted, to yield a cross-linking percentage of 87%. The amount of lipase immobilized reached 0.140 kg/kg_{fiber}. Lipase adsorbed during ionic interaction with the grafted polymer chains was eluted by the 0.5 M NaCl solution. However, 35% of the epoxy groups in the grafted polymer chains remained in the DEA fiber.

The degree of multilayer binding of the lipase was calculated by dividing the amount of lipase immobilized by the theoretical monolayer binding capacity, q_t:

$$q_t = \frac{a_v M_r}{a N_A} \quad [10]$$

where a_v and a are the specific surface area of the DEA fiber (4300 m^2/kg_{fiber}) and the area occupied by the lipase [2.6 × mass of lipase (27,000) (15)], respectively. For the lipase, q_t was calculated as 0.074 kg/kg_{fiber}. The degree of multilayer binding of the lipase fiber was 20.

Esterification reaction using immobilized lipase. When lipase fiber was used in the batch reactor, the lipase activity was 0.53 mol/(h·kg_{lipase}). When lipase fiber was reused five times after being used in the batch reactor for 24 h, lipase activity showed no signs of denaturation. However, when lipase fiber without glutaraldehyde cross-linking was used, lipase activity decreased after reuse. Lipase probably desorbed from the micropores of the hollow fibers without cross-linking. Native lipase activity was 0.38 mol/(h·kg_{lipase}) in the batch reactor. When the native lipase and hollow fiber without immobilized lipase were added to the reaction mixture, the lipase activity was almost the same value. Thus, lipase activity was increased by immobilization with an ion-exchange group containing a

grafted polymer brush and cross-linking. The effect of the polymer chain coating on the enzyme was thought to be the same as that of the hydrophobic chain of a surfactant.

The reaction percentage defined by Equation 8 is shown in Figure 5 as a function of the SV of a substrate solution. SV ranging between 61 and 420 h^{-1} correspond to a residence time of 60 to 7.5 s. The reaction percentage decreased exponentially with increasing SV; at an SV of 68 h^{-1}, the highest degree of conversion reached was 50%.

Because this reaction produced water, we expected that water might affect the reaction. However, when the reaction was carried out as a batch reaction, the conversion reached greater than 98% at equilibrium; thus, we did not consider water to inhibit esterification in this study.

Immobilized enzyme activity as a function of SV of the substrate solution is shown in Figure 6. Immobilized enzyme activity increased gradually with increasing SV. At an SV of 480 h^{-1}, immobilized enzyme activity was 8.9 mol/($h \cdot kg_{lipase}$), which was 23.4 times higher than that of native lipase activity [0.38 mol/($h \cdot kg_{lipase}$)]. Lipase immobilized with a polymer brush on the membrane was activated by multilayering and coating effects, and the convective flow of the substrate solution through the lipase fiber induced a higher enzymatic reaction.

The lipase fiber reaction occurred *via* three steps: Step 1: convective transport of substrates near the enzyme layer; Step 2: diffusional mass transfer of fatty compounds into the grafted chains, immobilizing the cross-linked lipases; and Step 3: intrinsic enzymatic reaction of the substrate at the active lipase site. Step 1 was not a rate-determining step because the time required for the convection-aided transport of the substrate was much shorter than the residence time of the substrate (16). Dependence of the reaction percentage on SV indicates that the latter two steps, i.e., diffusion of the substrate and the subsequent reaction, govern the overall enzymatic reaction rate, although the contribution of each process to the reaction rate remains unclear.

In this study, we determined that this immobilization method was an effective measure for protecting enzyme activity in a hydrophobic solvent. This system may be applicable to TG methanolysis (biodiesel production), which produces no water.

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