Efficient Preparation of (*R*)-α-Monobenzoyl Glycerol by Lipase Catalyzed Asymmetric Esterification: Optimization and Operation in Packed Bed Reactor

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Abstract: Optically active (*R*)-α-monobenzoyl glycerol (MBG) was synthesized by *Candida antarctica* lipase B (CHIRAZYME® L-2) catalyzed asymmetric esterification of glycerol with benzoic anhydride in organic solvents. Various conditions, such as the type and composition of the organic solvent, water content of the system, reaction temperature, and concentrations of the substrates were systematically examined and optimized in screw-capped test tubes with respect to both the reaction rate and the enzyme selectivity. 1,4-Dioxane was found to be the best solvent and no additional water was needed for the system. The optimum temperature was around 30°C, while the most suitable substrate concentrations were 100 mM each for glycerol and benzoic anhydride, respectively. However, when excessive anhydride (e.g., 200 mM) was used, the produced MBG could be further transformed into 1,3-dibenzoyl glycerol (DBG) by the same enzyme with a priority to (*S*)-MBG, resulting in a significant improvement of the product optical purity from ca. 50–70% *e.e.* Under optimal conditions (100 mM glycerol, 100–200 mM benzoic anhydride, dioxane, 25–30°C), the enzymatic synthesis of (*R*)-MBG was successfully operated in a packed-bed reactor for about 1 week, with an average productivity of 0.79 g MBG/day/g biocatalyst in the case of continuous operation and 0.94 g MBG/day/g biocatalyst in the case of semicontinuous operation. After refinement and preferential crystallization of the crude product, (*R*)-MBG could be obtained in an almost optically pure form (>98% *e.e.*). *Biotechnol Bioeng* **73**: 493–499, 2001.

Keywords: *Candida antarctica* lipase B; asymmetric esterification; (*R*)-α-monobenzoyl glycerol; benzoic anhydride; monoglyceride; packed bed reactor

INTRODUCTION

Optically active monoglycerides, as one class of important chiral C3 synthons, are widely used in the preparation of many enantiomerically pure medicines, agrochemicals, and liquid crystals (Kloosterman et al., 1988). Lipases, although classified as triacylglycerol hydrolases (EC 3.1.1.3), have currently been employed more frequently as powerful tools for selective transformation of unnatural substrates than as merely catalysts for splitting fats and oils (Schmid and Verger, 1998; Jaeger and Reetz, 1998). For example, optically active acids, alcohols, amines, and their esters or amides could be prepared by lipase-catalyzed enantioselective acylation, deacylation, or acyl transfer reactions (Chen and Sih, 1989; Dordick, 1989; Klibanov, 1990; Faber and Riva, 1992; Santaniello et al. 1993; Roberts, 1999). In all these cases, however, the desired enantiomers are usually obtained in yields of less than 50%, unless the undesired enantiomers could be racemized by other methods. We have reported a novel one-step method (Kato et al., 1999, 2000), with a theoretical yield of 100%, for the synthesis of optically active α-monobenzoic glycerol (MBG) by lipase-catalyzed asymmetric acylation of prochiral glycerol with vinyl benzoate or benzoic anhydride in organic solvents (Scheme 1).

Stereoselectivity is perhaps the most attractive feature of enzymatic synthesis, although it is not always satisfactory for all reactions, especially for those of unnatural substrates. A number of factors have been found that can affect the enantioselectivity of enzyme-catalyzed reactions. They include site-directed mutagenesis (Ozaki and Ortiz de Montellano, 1994), alternation of reaction conditions such as pH (Liu et al., 1999), temperature (Phillips, 1992), solvent (Tawaki and Klibanov, 1992), and use of additives (Guo and Sih, 1989; Colton et al. 1995; Itoh et al., 1996; Liu et al., 2000). However, very little is known about improving the prochiral selectivity of lipase-catalyzed asymmetric synthesis (Terradas et al., 1993). On the other hand, although examples of biocatalytic synthesis in organic solvent systems have been well documented, few were optimized and operated in bioreactors in spite of their importance for large-scale preparations (Kyotani et al., 1988; Nakanishi et al.,

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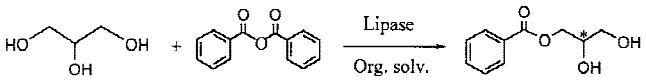

Scheme 1. Asymmetric esterification of glycerol with benzoic anhydride catalyzed by lipase in organic solvent.

1990; Xu et al., 1995b, Kline et al., 2000). In this article the optical purity of enzymatically synthesized MBG was found to be dependent on concentration of the acyl donor (benzoic anhydride) and this mechanism is revealed for the first time. Furthermore, after systematic optimization of reaction conditions we were able to operate the lipase-catalyzed asymmetric esterification of glycerol efficiently in a packed-bed reactor, affording the product of (*R*)-MBG in a very nice form (colorless needle crystal) and with high optical purity (>98% *e.e.*).

MATERIALS AND METHODS

Enzyme and Chemicals

A lipase from *Candida antarctica* (fraction B), either in a free form (CHIRAZYME L-2, lyo.) or fixed on a carrier (CHIRAZYME® L-2, c.-f., C2, lyo.), was purchased from Roche Diagnostics (Tokyo, Japan) and used throughout this work without further treatment. Glycerol (G.R.) was from Nacalai Tesque (Kyoto, Japan), benzoic anhydride (E. P.) was from Tokyo Kasei (Tokyo, Japan), and 1,4-dioxane (E. P.) was from Wako Pure Chemicals Industry (Osaka, Japan). All other chemicals were also from commercial sources and of analytic grade. Authentic (*S*)- and (*R*)-MBG were synthesized as described by Yodo et al. (1988).

Enzymatic Reactions on Shaker

For optimization of reaction conditions, enzymatic reactions were usually performed in screw-capped test tubes on a reciprocal shaker (170 rpm). In a typical reaction, 10 mg of carrier-fixed Chirazyme L-2 (c.-f., C2, lyo.) and 0.20 g of anhydrous Na_2SO_4 was added to 2 ml of dioxane solution containing 50 mM each of glycerol and benzoic anhydride, and the mixture was shaken at 170 rpm and 30°C.

HPLC Analysis

(*S*)- and (*R*)-MBGs were determined by HPLC (Shimadzu) using a CHIRALPAK AD-RH column (ϕ4.6 × 150 mm; Diacel Chemical Industries, Japan) eluted with a mixture of methanol and 5 mM H_3PO_4 (60:40, v/v). To remove the benzoic anhydride remaining in the reaction mixture, 100 μl of sample was first mixed with 100 μl of 10 mM benzamide solution (with 1% DMSO) as an internal standard and extracted twice with 100 μl each of petroleum ether. Then the aqueous phase was mixed with 100 μl of a combined solution with 4 M NaCl and 0.2 M $NaHCO_3$ to remove the benzoic acid formed (whose HPLC peak was overlapped by that of (*R*)-MBG) and finally MBG was extracted into 100 μl of ethyl acetate which was subjected to HPLC analysis after being properly diluted (50–100-fold).

Enzyme Reactor and Operation

Into an empty HPLC column (ϕ0.8 × 30 cm, bed volume: 15 ml) was packed 5.0 g of carrier-fixed CHIRAZYME L-2. The enzyme column was put into an incubation box and connected with an HPLC pump (LC-6A, Shimadzu, Kyoto). For continuous operation of the reactor, a reaction mixture (100 mM glycerol and 100–120 mM benzoic anhydride in dioxane) was pumped into the enzyme column at varying flow rates (0.3–0.1 ml/min). Since deactivation of the enzyme is inevitable, measures were taken to keep the conversion ratio of glycerol at a certain level (around 70%). These measures include elevation of reaction temperature (27–32°C), increase of anhydride concentration (100–120 mM), and reduction of flow rate (0.3–0.1 ml/min). In the case of semicontinuous operation, 500 ml of reaction mixture was recycled through the enzyme column at a high flow rate of 120 ml/h. After one batch of the reaction was completed the enzyme column was washed by 50 ml of dioxane and then the next batch of semicontinuous reaction was started by recycling a newly prepared substrate solution (500 ml).

Product Refinement

The reaction mixture was evaporated under reduced pressure for removal of dioxane and the residue was mixed with saturated brine and extracted by ethyl acetate three times. The combined organic layer was successively washed with 5% $NaHCO_3$ solution and saturated brine and dried over anhydrous Na_2SO_4. After evaporation of the organic solvent, the crude MBG was applied onto a silica gel column and eluted by *n*-hexane/ethyl acetate (1:1). One gram of the refined MBG was dissolved in 5 ml of 2-propanol at 60–65°C and to this solution was slowly added 95 ml of *n*-hexane. The hot solution was cooled at room temperature and (*R*,MBG was added as a seed to initiate the preferential crystallization. After the initial crystal appeared the mixture was further chilled overnight at −20°C, then the crystal formed was filtrated, washed with hexane/2-propanol (95:5), and finally dried over silica gel under reduced pressure.

RESULTS AND DISCUSSION

Enzymatic Glycerolysis and Hydrolysis of Benzoic Anhydride

Lipase-catalyzed ester synthesis or hydrolysis is known to proceed via an acyl-enzyme intermediate with which both an alcohol or water can act competitively as nucleophiles (Xu et al., 1995a). Therefore, it is necessary to make a comparison between these two parallel reactions (glyceroly-

sis and hydrolysis) of the anhydride. At first the simultaneous chemical glycerolysis of the highly reactive benzoic anhydride, which may occur during the enzymatic process and thus decrease the optical purity of MBG formed, was examined in a control experiment without any biocatalyst. As shown in Fig. 1 the chemical acylation of glycerol was practically negligible, only about 1.5% conversion after 120 h of incubation at 30°C. When this reaction was catalyzed by CHIRAZYME L-2 (*Candida antarctica* lipase, fraction B) in either free (5 mg) or carrier-fixed (10 mg) form, the initial rates in both cases were enhanced by approximately 160-fold as compared with that of control. However, the enantiomeric excess (*e.e.*) of MBG produced by the free enzyme was significantly lower than that by the carrier-fixed enzyme, although the reason for this difference is not yet clear. Therefore, the carrier-fixed lipase was chosen as the biocatalyst for the subsequent experiments.

The degradation of benzoic anhydride into its corresponding free acid was also examined under a similar condition as for glycerolysis by omitting glycerol (data not shown). As a result, the hydrolytic reaction of anhydride occurred only in the presence of free or carrier-fixed CHIRAZYME L-2, indicating that it was not simultaneous but catalyzed by the enzyme. Furthermore, the rate of anhydride hydrolysis was three times higher than that of glycerolysis even in the system without additional water. This suggests that partial hydrolysis of anhydride is practically inevitable, which is easy to understand from the acyl-enzyme mechanism of hydrolases (Xu et al., 1995a).

Effect of Water on Enzymatic Esterification

To investigate the effect of water content on the enzymatic synthesis of MBG, various amounts of water were added to the reaction system (Fig. 2). As predicted, additional water was indeed detrimental to the production of MBG because water facilitates the hydrolysis of benzoic anhydride into free benzoic acid, whose reactivity as an acyl donor was much lower (ca. 180 times) than that of benzoic anhydride. Furthermore, the influence of water addition on the stereoselectivity of the lipase was not obvious since the effect on the rates of (*S*)- and (*R*)-MBG formation were very similar. In fact, the formation of (*S*)-MBG was always ca three times faster than that of (*R*)-MBG in all water contents tested. Therefore, for efficient production of MBG with benzoic anhydride as the acyl donor it is essential to keep the water content of the reaction system as low as possible.

On the other hand, some water-removing additives, such as anhydrous sodium sulfate and activated molecular sieve, were introduced into the reaction system to check if the trace amount of water still in the system is excessive for enzymatic esterification and needed to be further removed. As a consequence, only slight differences were observed between the reaction with either anhydrous sodium sulfate or activated molecular sieve and that without any water-removing additives (data not shown), indicating that the residual water in the system was optimum for the enzymatic reaction. Nevertheless, an adequate amount of anhydrous Na_2SO_4 should be added as a buffering agent of water activity (Kuhl and Halling, 1991) to prevent any possible invasion of water from the air into the reaction system.

Comparison of Various Solvents

For enzymatic asymmetric synthesis of MBG in an organic solvent system, the composition of the solvent system is also an important factor for careful optimization because the medium of reaction can affect not only the enzyme activity but also the enantioselectivity or prochiral selectivity (Tawaki and Klibanov, 1992; Terradas et al., 1993; Xu et al., 1998). Taking into consideration both the highly hydrophobic anhydride and the highly hydrophilic glycerol, dioxane was chosen as a good solvent for both substrates. For

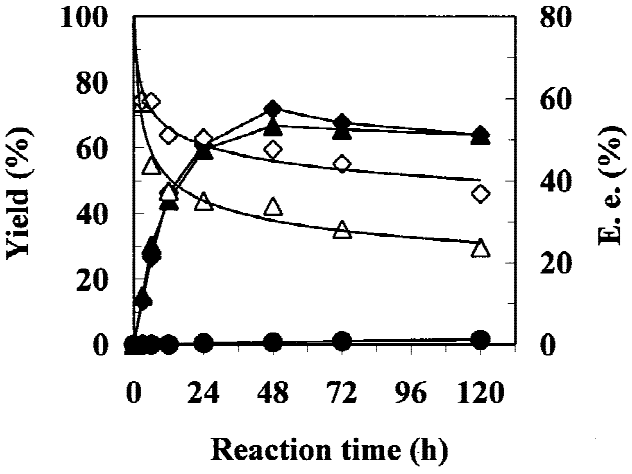

Figure 1. Lipase-catalyzed glycerolysis of benzoic anhydride, forming α-monobenzoyl glycerol (MBG). Enzymatic reactions were carried out on a reciprocal shaker (30°C, 170 rpm) by adding 5 mg of free Chirazyme L-2 (△, ▲) or 10 mg of carrier-fixed Chirazyme L-2 (◇, ◆), while in the case of control (●), no enzyme was added. The reaction mixture was composed of 100 μmol benzoic anhydride, 100 μmol glycerol, and 2 ml dioxane. Symbols: ●▲◆, yields of MBG; △◇, enantiomeric excesses of (*R*)-MBG.

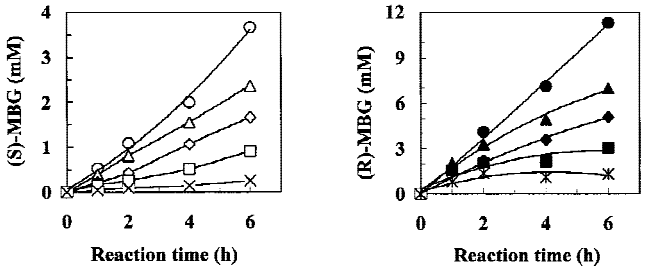

Figure 2. Enantioselective formation of (*S*)- and (*R*)-MBG by lipase-catalyzed monobenzoylation of glycerol in dioxane with various water content. Reactions were performed on a reciprocal shaker (30°C, 170 rpm) by adding 10 mg of the carrier-fixed Chirazyme L-2 into a mixture containing 100 μmol glycerol, 100 μmol benzoic anhydride, 2 ml dioxane, and various amounts of water added (%, v/v): 0 (○●): 0.1 (△▲); 0.3 (◇◆); 0.5 (□■); or 1.0 (× *). For clarity, the time courses of (*S*)- and (*R*)-MBG formation in one reaction system are plotted separately.

comparison of various solvents in a homogenous phase, the enzymatic reaction was carried out in a mixed solvent system composed of a half volume of 1,4-dioxane and a half volume of another solvent. Anhydrous sodium sulfate was added into the system to keep water activity at an identical level. The results of lipase-catalyzed reactions in 14 mixed solvent systems are shown in Figure 3.

The enzymatic reaction in systems containing highly hydrophobic alkanes, such as isooctane, *n*-hexane, and cyclohexane, showed relatively high rates but lower enantioselectivity as compared with those in 100% dioxane. Diisopropyl ether (IPE) and methyl isobutyl ketone (MIBK) gave similarly good results. Acetone, acetonitrile, and dimethyl sulfoxide (DMSO) showed significant denaturing effects on the enzyme, although the enzyme selectivity in these highly hydrophilic solvents were little better than that in 100% dioxane. Furthermore, the volume fractions of some promising systems containing dioxane and isooctane, *n*-hexane, IPE, or MIBK were altered to search for an optimum composition of the mixed solvent. As a result (data not shown), a decrease in dioxane content of the mixed solvent system containing highly hydrophobic isooctane or *n*-hexane resulted in an obvious decrease in the stereoselectivity of the enzyme, while only slight a drop of enzyme selectivity was observed in the system with MIBK or IPE. In all these systems, the enzyme activities also decreased with the reduction of dioxane fractions to some extent indicating the suitability of 100% dioxane as the solvent for the lipase-catalyzed mono-benzoylation of glycerol with respect to either yield or *e.e.* of MBG.

Effect of Reaction Temperature

Figure 4 shows the time-courses of MBG formation at different temperatures in the system of 100% dioxane. One can see that the reaction at 15°C was much slower (ca. 3-fold) than that at 30°C, while the optical purity of MBG formed was quite similar (around 50% *e.e.*). However, temperatures higher than 30°C showed little enhancing effect on the reaction rate, but considerable detrimental effects on both the stability and the selectivity of the enzyme in dioxane. Therefore, the enzymatic reaction should be operated at temperatures around or slightly below 30°C for activity, selectivity, and stability.

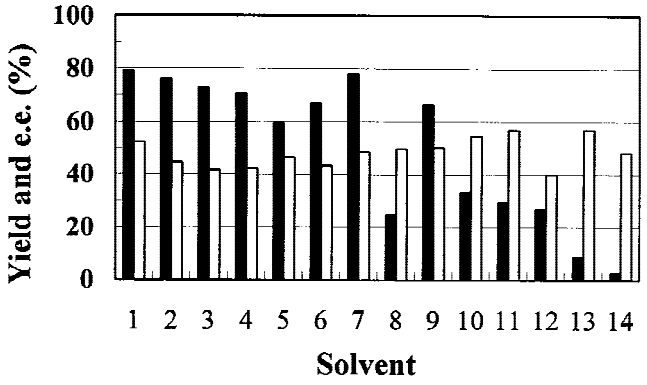

Figure 3. Lipase-catalyzed esterification in mixed-solvent systems containing 50% (v/v) of 1,4-dioxane and 50% of another solvent: 1) 1,4-dioxane; 2) isooctane; 3) *n*-hexane; 4) cyclohexane; 5) toluene; 6) *t*-butyl methyl ether; 7) diisopropyl ether; 8) chloroform; 9) methyl isobutyl ketone; 10) acetone; 11) acetonitrile; 12) tetrahydrofunan; 13) dimethyl sulfoxide; 14) *N*,*N*-dimethylformamide. Symbols: filled bar, yield of MBG (%); open bar, enantiomeric excess (%) of (*R*)-MBG formed. Reactions were performed at 30°C and 170 rpm for 24 h by adding 10 mg of carrier-fixed Chirazyme L-2 into a mixture of 100 μmol glycerol, 100 μmol benzoic anhydride, 0.20 g anhydrous Na_2SO_4, 1 ml dioxane, and 1 ml another solvent.

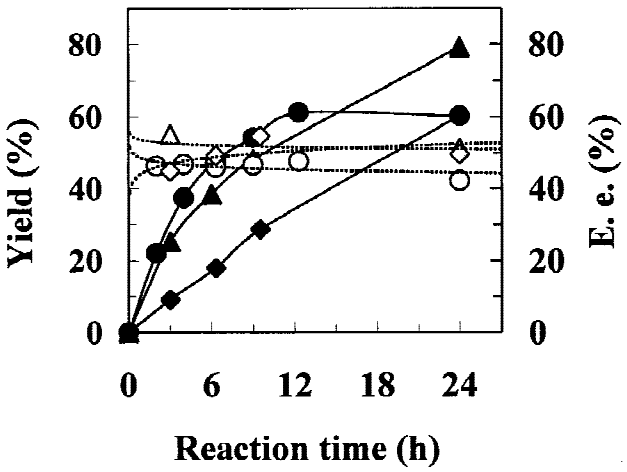

Figure 4. Effect of reaction temperature on yield (●▲♦) and enantioemeric excess (○△◇) of α-monobenzoyl glycerol in 1,4-dioxane. Temperatures: 40°C (●○); 30°C (▲△); 15°C (♦ ◇). The reaction mixture was composed of 100 μmol glycerol, 100 μmol benzoic anhydride, 10 mg of Chirazyme C-2, 0.20 g anhydrous Na_2SO_4, and 2 ml solvent.

Although neat dioxane will freeze at temperatures below 12°C, a mixed solvent of 75% dioxane and 25% *n*-hexane allowed us to perform the enzymatic reactions at temperatures down to 5°C. The reactions proceeded almost linearly until ca. 50% conversion (data not shown), indicating that the inactivation of enzyme under such relatively low temperatures (≤30°C) was not significant. In addition, few changes in the optical purity of MBG were observed in the range of 5–30°C, suggesting that enzyme selectivity cannot be further enhanced by lowering reaction temperature.

Dependence on Substrate Concentrations

The concentration effects of two substrates, glycerol and benzoic anhydride, on both the reaction rate and the prochiral selectivity of MBG synthesis are shown in Figure 5. When fixing the anhydride concentration at 100 mM and increasing the glycerol concentration from 50–200 mM (Fig. 5A), the initial rates observed were almost the same, indicating a very low *K*m of the *Candida antarctica* lipase B for its natural substrate (glycerol); the MBG produced started to decrease after 30 h, probably due to a chemical (benzoic acid-catalyzed) hydrolysis of MBG after anhydride was exhausted, since the *e.e.* of residual MBG was kept unchanged. Furthermore, the *e.e.* of MBG formed in the presence of high glycerol concentration was a little lower

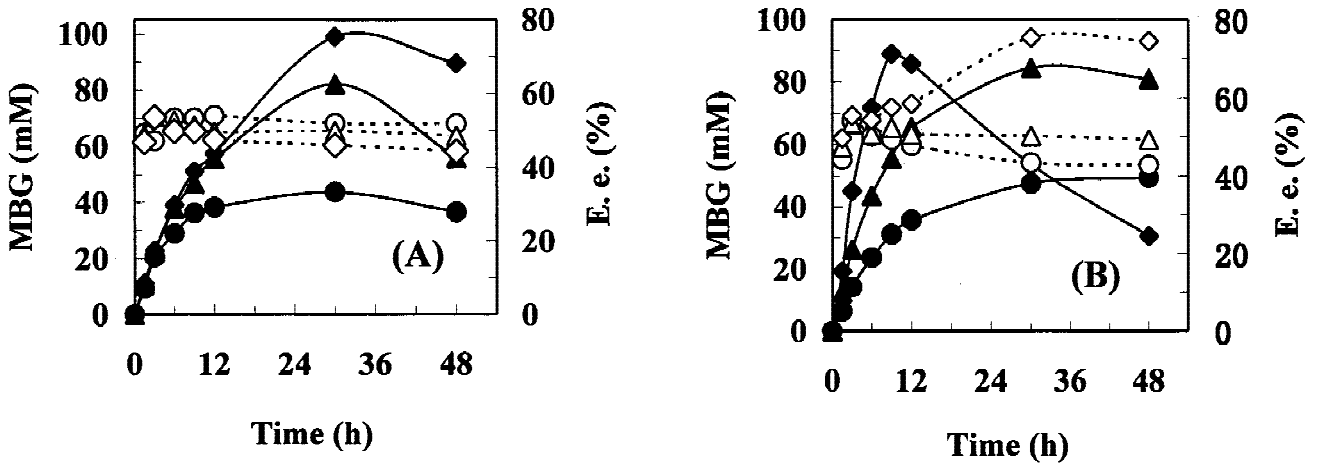

Figure 5. Dependence of MBG formation on the substrate concentration. Enzymatic reactions were carried out at 30°C and 170 rpm by adding 10 mg of carrier-fixed Chirazyme L-2 and 0.20 g of anhydrous Na_2SO_4 into 2 ml of dioxane solution containing; **A)** 100 mM of benzoic anhydride and 50 mM (●○), 100 mM (▲△) or 200 mM (♦ ◇) of glycerol; **B)** 100 mM of glycerol and 50 mM (●○), 100 mM (▲△), or 200 mM (♦ ◇) of benzoic anhydride. Symbols: ●▲♦, MBG concentrations; ○△◇, enantiomeric excesses of (*R*)-MBG.

than that in the case of less glycerol because excessive glycerol might facilitate the spontaneous glycerolysis (or the 1,3-site shift of the acyl group) of MBG, which might lead to its partial racemization.

On the other hand, when fixing the glycerol concentration at 100 mM and increasing the benzoic anhydride from 50–200 mM (Fig. 5B), it was observed that not only the reaction rate was enhanced, as can be expected, but, very interestingly, the *e.e.* of MBG was also significantly improved, especially in the presence of excessive anhydride (200 mM), where the optical purity of (*R*)-MBG increased gradually from 60–75% *e.e.* after ca. 9 h, accompanying a rather rapid decrease in MBG concentration.

To clarify the mechanism of enantioselective enrichment of (*R*)-MBG in the presence of excessive anhydride, a mixture of racemic MBG, benzoic anhydride, and the enzyme in dioxane was shaken at 30°C for 72 h and both the concentration and the *e.e.* of the residual MBG were monitored by HPLC. As shown in Figure 6, the racemic MBG could indeed be enzymatically resolved into (*R*)-MBG, with ca. 27% *e.e.* at 50% yield (around 12 h) and 55% *e.e.* at 18% yield (48 h). In addition, the product formed in this "side" reaction had been isolated by TLC and identified by HPLC and NMR as 1,3-dibenzoyl glycerol (DBG). The above results demonstrate that in the presence of benzoic anhydride the enzymatically produced MBG may be further transformed into DBG, with a priority to (*S*)-MBG, resulting in an enrichment of (*R*)-MBG in the residual product. This mechanism may be useful for control of the reaction time and for improvement of *e.e.* of the product.

Continuous Synthesis of MBG in a Packed-Bed Reactor

For a preparative synthesis of MBG and repetitive use of biocatalyst, a packed-bed reactor was constructed by filling the carried-fixed CHIRAZYME L-2 into an empty HPLC column. The reaction was started by continuously pumping the substrate solution (100 mM glycerol + 100 mM anhydride) at a flow rate of 0.3 ml/min through the enzyme column, which was incubated initially at 28°C in a temperature-controlled air box. Samples of the transformed solution from the outlet of the enzyme column were periodically withdrawn for monitoring the product concentration and *e.e.* value. When the conversion ratio of glycerol dropped to ca. 70%, the reaction temperature was elevated, by 1°C each time, up to a limit of 32°C. In this way the reaction could be maintained at a high level of conversion (>70%) for 2 days, with about 50% *e.e.* for the produced (*R*)-MBG (Fig. 7). On the third day, the flow rate of substrate solution was reduced to 0.2 ml/min, while the reaction temperature was decreased to 27°C at first and then increased stepwise to 29°C to keep

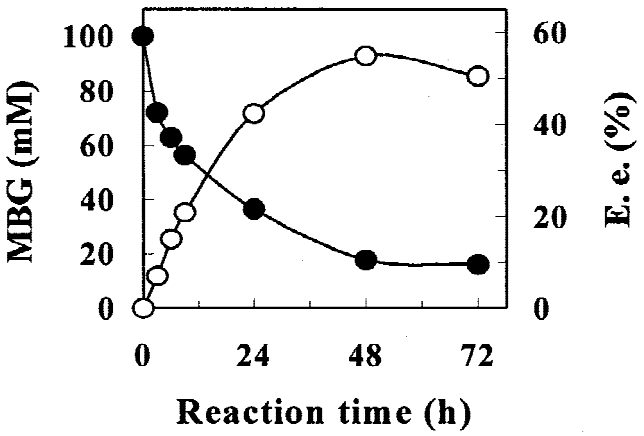

Figure 6. Decrease in (*R,S*)-MBG concentration (●) and increase in *e.e.* value of (*R*)-MBG (○) due to enantioselective transformation into dibenzoyl glycerol in the presence of benzoic anhydride and carrier-fixed Chirazyme L-2. Reaction conditions: (*R,S*)-MBG, 100 mM; benzoic anhydride, 100 mM; dioxane, 2 ml; enzyme, 10 mg; 30°C, 170 rpm.

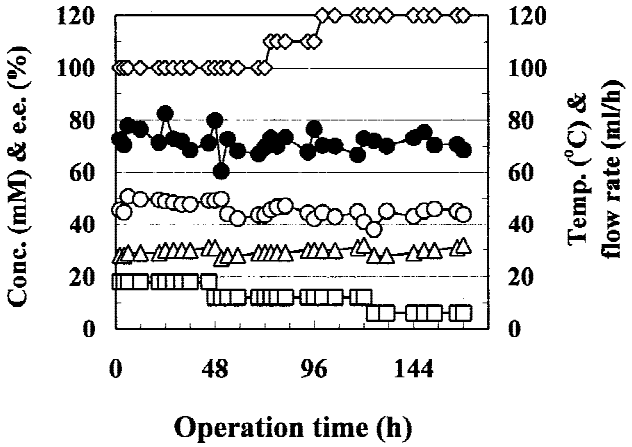

Figure 7. Continuous operation of enzymatic esterification in a column reactor packed with carrier-fixed Chirazyme L-2. Reaction conditions: carrier-fixed Chirazyme L-2, 5.0 g; working volume, 15.0 ml (ϕ0.8 × 30 cm); solvent, dioxane; glycerol, 100 mM; benzoic anhydride, 100–120 mM; flow rate, 18–6 ml/h; temperature, 27–32°C. Symbols: (●) MBG concentration; (○) *e.e.* of (*R*)-MBG; (△) reaction temperature; (□) flow rate of substrate solution; (△) initial concentration of benzoic anhydride.

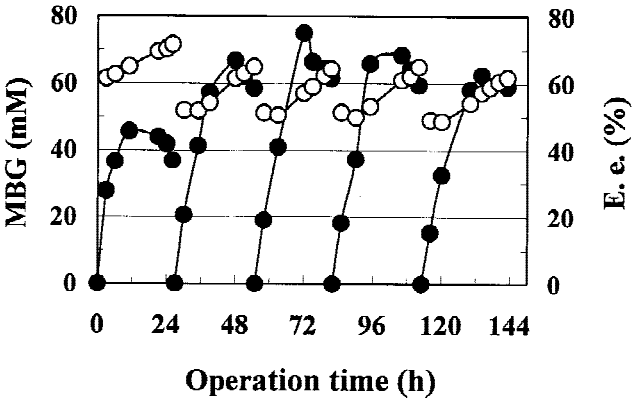

Figure 8. Semicontinuous operation of the enzyme reactor. For each batch of reaction, 500 ml of substrate solution was recycled at a flow rate of 120 ml/h through the column reactor packed with 5.0 g of carrier-fixed Chirazyme L-2. The substrate solution was composed of 100 mM glycerol and 200 mM benzoic anhydride in the case of the first batch, or 100 mM anhydride at the beginning of reaction and another 100 mM of anhydride supplemented after 8 h of reaction for batches 2–5. The temperature of reactions were controlled at: 25°C (batch 1), 25°C (batch 2), 27°C (batch 3), 28°C (batch 4), and 30°C (batch 5). Symbols: (●) concentration of MBG; (○) *e.e.* of (*R*)-MBG.

the conversion ratio around 70%. It is worth noting that there was little drop in the optical purity of MBG after the flow rate was reduced, probably due to the elongation of average residence time of substrate solution in the reactor, which may partially result in the nonselective chemical esterification of glycerol. On the fourth and fifth days, the concentrations of benzoic anhydride were increased to 110 mM and 120 mM, respectively. On the sixth and seventh days, the flow rate was further reduced to 0.1 ml/min to maintain the desired conversion of glycerol. As a result of this "fuzzy" control of temperature, flow rate, and anhydride concentration, the enzymatic synthesis of (*R*)-MBG in the column reactor was continuously operated for 1 week, giving quite high and stable yields (around 70%) but somewhat low optical purity (50–40% *e.e.*). The average productivity of the reactor was estimated to be 169 μmol MBG·h^{-1}·g $biocatalyst^{-1}$ or 0.79 g MBG·d^{-1}·g $biocatalyst^{-1}$.

Preparation of (R)-MBG in Semicontinuous Operation of the Enzyme Reactor

In order to avoid too-complicated control in the continuous operation, the packed-bed reactor was alternatively operated in a semicontinuous mode, i.e., by fast recycling of substrate solution through the enzyme column at a flow rate of 2 ml/min, which had been optimized in a preliminary test. To improve the optical purity of (R)-MBG produced, a twice higher concentration (200 mM) of benzoic anhydride was employed for the enzymatic acylation of glycerol (100 mM). As shown in Figure 8, the first run of the reaction with 200 mM of anhydride afforded (*R*)-MBG in the highest *e.e.* (71.5%) but a rather low yield (36.8%) at the endpoint of reaction (26 h). This might result from a shortage of the water necessary for enzyme function caused by the water-consuming hydrolysis of benzoic anhydride, especially in the presence of largely excessive anhydride (Xu et al., 1995a). Therefore, the required amount (200 mM) of anhydride was divided into two equal parts and fed separately to the substrate reservoir at 0 h and 8 h in the subsequent runs of the reaction (batch No. 2-5). As a consequence, the lipase-catalyzed esterification proceeded more smoothly than the first run and was terminated at ca. 60% yield after passing over a peak of MBG concentration, giving (*R*)-MBG in 62–65% *e.e.*, which was never reached in our previous studies (Kato et al., 1999, 2000). Furthermore, the calculated productivity of the enzyme reactor in semicontinuous operation (0.94 g MBG·d^{-1}·g $biocatalyst^{-1}$, as an average of batch 2-5) was also higher than that obtained in the continuous operation (0.79 g MBG·d^{-1}·g $biocatalyst^{-1}$). Finally, a colorless crystal (needle type) of (*R*)-MBG was successfully prepared after a simple work-up of the reacted mixture, with a total yield of 26–34% (based on the amount of glycerol) and an optical purity of 92–95% *e.e.*, which could be further enhanced to >98% *e.e.* by preferential recrystalization of the first crystal, usually with a satisfactory yield of around 80%.

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