JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Production of Ascorbyl Palmitate by Surfactant-Coated Lipase in Organic Media

HSIN-JU HSIEH,† GIRIDHAR R. NAIR,‡ AND WEN-TENG WU*,‡

Department of Chemical Engineering, National Tsing Hua University, Hsinchu, Taiwan, and Department of Chemical Engineering, National Cheng Kung University, Tainan, Taiwan

The surface of a lipase from *Burkholderia cepacia* was coated with a nonionic surfactant, propylene glycol monostearate, and was used as a biocatalyst in the production of ascorbic acid in *tert*-butyl alcohol. The influence of various factors such as the type of surfactant, the pH of the buffer used for coating, the amount of surfactant in the coating, the organic solvent, and the temperature and molar ratio of the substrates used in the reaction on the conversion of ascorbyl palmitate were studied. After 24 h of reaction at 50 °C, a conversion of 47% was obtained using an ascorbic acid to palmitic acid molar ratio of 1:6. The native lipase showed only 6% conversion.

KEYWORDS: Acylation; antioxidant; ascorbyl palmitate; surfactant-coated lipase

INTRODUCTION

Ascorbyl palmitate (6-*O*-palmitoyl-L-ascorbic acid) is an amphipathic ester of ascorbic acid (vitamin C), which is used widely in food, cosmetics, and pharmaceutics because of its antioxidant properties (*1*). Commercially, it is synthesized by the acid-catalyzed acylation of ascorbic acid. Besides being energy-intensive, chemical synthesis often leads to the formation of a mixture of products that makes the purification difficult (*2*). Enzyme-catalyzed acylation of ascorbic acid using palmitic acid or its esters as the acyl donor has been developed as an alternate method of the synthesis of ascorbyl palmitate (*1*−*6*). Mild reaction conditions, higher yields, and purer products are the advantages of the enzymatic synthesis.

Enzymatic synthesis using lipase in nonaqueous organic solvents has several advantages. The solubility of nonpolar substrates is increased in organic solvents, and the reaction direction can be shifted to favor synthesis over hydrolysis. However, like all other natural enzymes, organic solvents easily denature lipase. To avoid the deactivation of the enzyme in organic media, modification of the surface of the enzyme by coating it with surfactants has been studied (*7*−*12*). The most significant advantages of the surfactant-coated enzymes are their simple preparation procedure, good solubility in a wide range of organic solvents, and better catalytic activity compared with the free enzyme (*8*, *9*).

Surfactant-coated lipases originating from various sources and coated with different surfactants have been used successfully as biocatalysts in a number of reactions of industrial importance. For example, *Mucor javanicus* lipase coated with glutamic acid dioleyl ester ribitol, which is a nonionic surfactant, has been used in the interesterification of triglycerides (*8*) and in the resolution of racemic ibuprofen (*9*). A lipase from *Pseudomonas cepacia* was coated with glutamic acid dioleyl ester ribitol amide and was used in the enzymatic ring-opening polymerization of lactones, which is one of the interesting routes to polymer-forming reaction (*10*). Similarly, a lipase from *Candida cylindracea* coated with Span 85, which is a polyether-type nonionic surfactant, was used to catalyze the esterification of geraniol and acetic acid (*11*). The esterification of long-chain fatty acids and fatty alcohols catalyzed with a biocatalytic complex produced by coating *Rhizopus japonicus* lipase with sorbitan monostearate has also been investigated (*12*). In all of the above cases the surfactant-coated lipases have been shown to exhibit higher catalytic activities compared to their native forms.

In the present study, a lipase from *Burkholderia cepacia* was coated with a nonionic surfactant and was used as a biocatalyst in the synthesis of ascorbyl palmitate in organic medium. Ascorbyl palmitate has been synthesized in the past using Novozym 435, which is a commercial immobilized lipase (*1*, *2*) and by using a thermostable lipase (*3*). The huge initial cost of such enzymes will make the ascorbyl palmitate very expensive. In contrast, the cost of ascorbyl palmitate produced by means of surfactant-coated lipase prepared by using inexpensive and food grade surfactants will be severalfold lower. Besides, because ascorbyl palmitate is widely used in foods, cosmetics, and pharmaceuticals, the use of food grade surfactants in place of synthetic surfactants in the coating of the enzyme will result in a safer product and greatly increase the "green" effect of the process. The utilization of surfactant-coated lipase for the enzymatic synthesis of vitamin C esters has not been studied until now. Furthermore, surfactant-coated forms of the lipase from this source have not been described in the literature.

* Corresponding author (telephone +886-6-237-6734; fax +886-6-275-4228; e-mail wtwu@mail.ncku.edu.tw).
† National Tsing Hua University.
‡ National Cheng Kung University.

10.1021/jf060089d CCC: $33.50
Published on Web 07/15/2006

Table 1. Surfactants Investigated for the Preparation of Coated Lipase

surfactant	source	hydrophilic part	lipophilic part	HLB[a]
sucrose stearate		sucrose	stearic acid	3
propylene glycol monostearate	Danisco Ingredients, Copenhagen, Denmark	propylene glycol	stearic acid	3.4
lactic acid glyceryl monostearate	Taiyo-Kagaku Co. Ltd., Mie, Japan	lactic acid	stearic acid	7.5
sorbitan monostearate	Yue Ba Enterprises Co. Ltd., Taipei, Taiwan	sorbitan	stearic acid	14.9

[a] Hydrophilic–lipophilic balance.

MATERIALS AND METHODS

Materials. *B. cepacia* (formerly *P. cepacia*) lipase was obtained from Amano Enzyme Inc. (Nagoya, Japan). Palmitic acid was obtained from Sigma (St. Louis, MO). Industrial grade ascorbic acid ($[\alpha]^{25}_D$ = +16°) was purchased from Camao International Co. Ltd. (Taipei, Taiwan). Reagent grade *tert*-butyl alcohol (maximum water content = 0.1%) was used as the solvent without further purification. Nonionic surfactants used for coating the enzyme were of food grade. All other chemicals used were of reagent grade.

Preparation of Surfactant-Coated Lipase. Surfactant-coated lipase was prepared according to the method of Basheer et al. (*12*) with a minor modification. Half a gram of lipase was dissolved in 100 mL of 0.05 M Tris−NaOH buffer at pH 6.4 and stirred at 4 °C for 20 min. A solution containing 0.05 g of surfactant in 5 mL of warm ethanol (40 °C) was added dropwise to the enzyme solution and sonicated for 10 min. The solution was then stirred for 3 h at 8−10 °C. The precipitate was collected by centrifugation and dried under vacuum. The surfactant-coated lipase obtained as a white powder was used as the biocatalyst in the preparation of ascorbyl palmitate. The yield of surfactant-coated lipase powder obtained was calculated as the percentage from the amount of lipase and surfactant initially taken.

General Procedure for Ascorbyl Palmitate Synthesis. A typical reaction mixture consisted of various molar ratios of ascorbic acid and palmitic acid in 10 mL of *tert*-butyl alcohol, 0.3 g of surfactant-coated lipase, and 0.5 g of molecular sieve (4 Å). The mixture was reacted at 50 °C for 24 h with continuous shaking at 180 rpm. Afterward, the mixture was filtered to remove the biocatalyst, and the filtrate was analyzed for the product. For a comparative study, acylation of ascorbic acid with palmitic acid in the presence of free lipase from *B. cepacia* was also carried out under the same conditions.

Analytical Methods. The ascorbic acid and ascorbyl palmitate were analyzed by HPLC (L-7100, Hitachi, Tokyo, Japan) equipped with a LiChroCART 250-4 Purospher RP-18e reversed-phase column (250 mm × 4 mm, Merck, Darmstadt, Germany) and a UV detector (SPD-6A, Shimadzu, Kyoto, Japan) at 292 nm. The mobile phase consisted of methanol/water/acetic acid (95:5:0.1, by vol) at 1 mL/min and 35 °C. The percentage conversion was calculated on the basis of the number of moles of ascorbic acid, which was the limiting substrate, reacted initially.

The catalytic activity of the surfactant-coated lipase was assayed in the hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP) as the substrate, according to the method described in our previous work (*13*). *p*-NPP was dissolved at a concentration of 0.5% (w/v) in reagent grade (95%) ethanol. The reaction mixture consisted of 1 mL of substrate, 1 mL of 0.05 M phosphate buffer (pH 7), and 0.2 g of surfactant-coated lipase. After 5 min of reaction at 30 °C, the reaction was terminated by adding 2 mL of 0.5 N Na_2CO_3. The mixture was centrifuged at 10000 rpm for 10 min, and the absorbance of the supernatant was measured at 410 nm in a Beckman DU 530 (Fullerton, CA) spectrophotometer. A molar extinction coefficient of 15000 M^{-1} cm^{-1} for *p*-nitrophenol was used (*14*). One unit of activity was defined as the amount of enzyme necessary to hydrolyze 1 μmol per minute of *p*-NPP under the conditions of assay. The protein content of the surfactant-coated lipase was estimated according to the method of Bradford (*15*) using bovine serum albumin (BSA) as a standard.

RESULTS AND DISCUSSION

Lipase from *B. cepacia* was coated with a nonionic surfactant, propylene glycol monostearate (PGMS90), and was used as an acyl transfer catalyst in the biosynthesis of ascorbyl palmitate in *tert*-butyl alcohol. Under optimized conditions, 47% of the ascorbic acid used in the reaction was converted to ascorbyl palmitate by the surfactant-coated lipase, whereas the native lipase showed only 6% conversion. The catalytic activity of the lipase was drastically increased upon coating it with the surfactant. Coating the native lipase with other nonionic surfactants (**Table 1**) having various hydrophilic−lipophilic balances (HLB) did not enhance its catalytic activity (data not shown). All of the surfactants used contained different hydrophilic portions, but with the same lipophilic moiety. It indicated that the activity of the surfactant-coated lipase was greatly influenced by the molecular structure of the hydrophilic region of the surfactant. The activity and stability displayed by the surfactant-coated lipase will be intrinsically connected to the type and structure of coating surfactant (*16*).

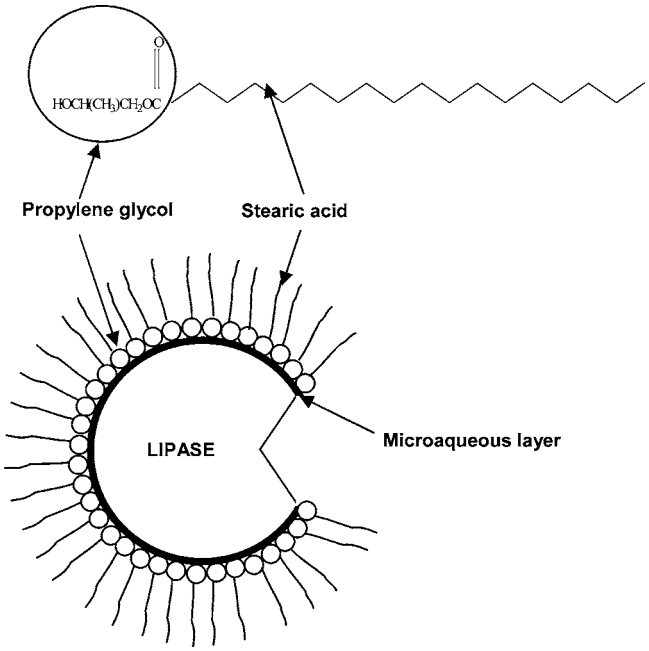

Figure 1. Schematic representation of lipase coated with propylene glycol monostearate. The hydrophilic propylene glycol moiety of the surfactant interacts with the enzyme surface, whereas the lipophilic monostearate chain is extended from the surface.

A model for the interaction of a surfactant with lipase has been proposed by Okahata et al. (*17*), who explain that the hydrophilic headgroups of lipid molecules interact with the enzyme surface, whereas the lipophilic alkyl chains extend away from its surface. On the basis of the explanation of the formation of lipid-coated lipase (*17*), a likely explanation for the formation of surfactant-coated lipase is given as follows. When lipase was coated with PGMS90 (HLB 3.4), the hydrophilic propylene glycol interacted non-covalently with the enzyme surface and the lipophilic monostearate chain pulled out from it, thus forming a micellar structure as illustrated in **Figure 1**. It was also observed that whereas the unmodified lipase was insoluble

Table 2. Protein Content, Yield, and Catalytic Activity of Surfactant-Coated Lipase

pH	protein content[a] (%)	SCL yield[b] (%)	catalytic activity[c] (%)	conversion (%)
5.4	7.7	80	105	43
5.8	12.5	71	114	44
6.4	5.5	63	172	47
6.8	7.7	70	140	46
7.4	3.3	64	82	38
7.8	3.0	76	93	32

[a] The protein content is expressed as the percentage by weight of protein in the SCL. [b] The yield of surfactant-coated lipase (SCL) complex is expressed as the percentage from 0.1 g of surfactant and 1.0 g of lipase as the starting materials. [c] The catalytic activity of surfactant-coated lipase is expressed as the percentage of that of the free lipase, which is taken as 100%.

in *tert*-butyl alcohol, the surfactant-coated lipase exhibited an enhanced solubility in *tert*-butyl alcohol at the temperature of reaction. Formation of the micelles stabilized the enzyme in the organic solvent, which otherwise would have denatured it by removing the microaqueous layer surrounding it.

The pH of the buffer used in the coating of the native lipase with the surfactant influenced the catalytic activity of the surfactant-coated lipase. An enzyme maintains its ionization state from the most recent aqueous solution to which it has been exposed (*18*). Therefore, the coating of lipase with the surfactant should be performed at the optimum pH to achieve maximum activity and enzyme stability. In the present study, surfactant coating of the lipase was carried out by using Tris−NaOH buffers of various pH (from 5.4 to 7.8). The protein content, yield, and catalytic activity of the surfactant-coated lipase and the percentage conversions of ascorbic acid to ascorbyl palmitate at different pH values are shown in **Table 2**. The protein content of the surfactant-coated lipase varied between 3.0 and 12.5% at different pH values. The yield of surfactant-coated lipase varied from 63 to 80% when the pH was changed from 5.4 to 7.8 during coating of the enzyme with the surfactant. The catalytic activity of the surfactant-coated lipase was highest (172%) when the pH was 6.4.

The amount of surfactant used for the coating of lipase had a profound effect on the catalytic activity of the surfactant-coated lipase. The coating was carried out using the conditions mentioned under Preparation of Surfactant-Coated Lipase. The relationship between the conversions obtained during synthesis and the weight ratios of surfactant and lipase used are shown in **Figure 2**. Highest conversion (47%) was obtained when a surfactant-to-lipase ratio of 1:10 was used (**Table 3**). A significant difference in the conversion was not observed at surfactant-to-lipase ratios lower than 1:10. However, lower conversions were obtained when surfactant-to-lipase ratios higher than 1:10 were used. At higher surfactant/lipase ratios the hydrophobic moiety of the free surfactant would form micellar structures with other surfactant molecules and move away from the enzyme surface or might form a bilayer around the enzyme molecule. In either case, a decrease in the enzyme activity can be expected.

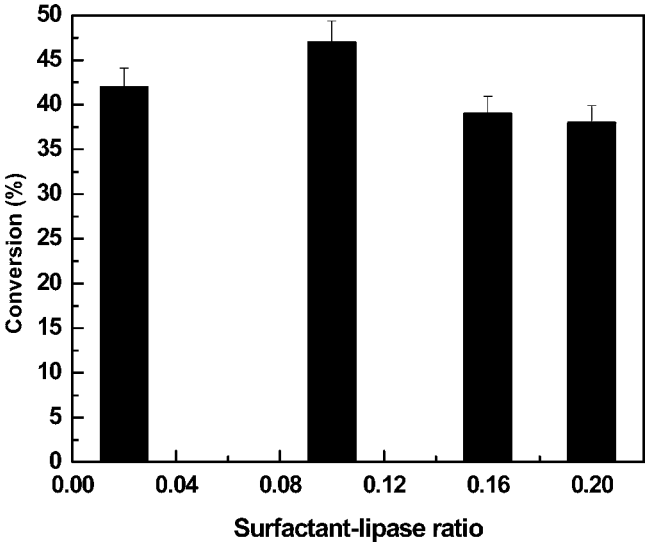

Figure 2. Effect of weight ratio of surfactant lipase used in the preparation of surfactant-coated lipase on the conversion.

The enzymatic activity of the surfactant-coated lipase in six different solvents (methanol, hexane, acetone, chloroform, *tert*-butyl alcohol, and isopropyl alcohol) was also investigated. The surfactant-coated lipase was completely inactive in all of the solvents except *tert*-butyl alcohol. It is well-known that lipases are generally inactivated in organic solvents. The sole cause of inactivation is the interaction between enzyme and solvent molecules that results in the stripping of the essential water molecule bound to the enzyme molecule. It causes a conformational change in the enzyme structure. Because the surfactant-coated lipase exhibited higher activity in *tert*-butyl alcohol, at the same time failing to show any activity in other solvents tested, it is evident that *tert*-butyl alcohol did not strip the essential water molecule bound to the enzyme. This microaqueous environment around the enzyme molecules has increased the stabilizing effect in the presence of the solvent. The higher catalytic activity exhibited by the surfactant-coated lipase can also be attributed to the higher solubility of the surfactant-coated lipase in *tert*-butyl alcohol. The increased lipophilicity due to the lower HLB of PGMS90 enhanced the solubility of the surfactant-coated lipase in *tert*-butyl alcohol. Furthermore, it has been established in our laboratory that the activity of immobilized lipase would be significantly increased in *tert*-butyl alcohol (*19*). Treatment with *tert*-butyl alcohol also regenerated the deactivated lipase (*19*). All of these factors have led to the higher catalytic activity of the surfactant-coated lipase.

The temperature of reaction had a profound effect on the catalytic activity of surfactant-coated lipase. Highest conversion was observed when the reaction was carried out at 50 °C. Above or below this temperature, the conversions were found to be very low. The molar ratios of ascorbic acid and palmitic acid

Table 3. Comparison of Lipase-Catalyzed Synthesis of Ascorbyl Palmitate

lipase	solvent	reaction temp/ time (°C)/(h)	ascorbic acid to palmitic acid molar ratio	drying	conversion (%)	ref
Novozym 435	*tert*-amyl alcohol	60/50	1:2	molecular sieve	86	*1*
Novozym 435	*tert*-amyl alcohol	56/50	1:5		56	*2*
surfactant-coated lipase	*tert*-butyl alcohol	50/24	1:6	molecular sieve	47	this study
free lipase	*tert*-butyl alcohol	50/24	1:6	molecular sieve	6	this study

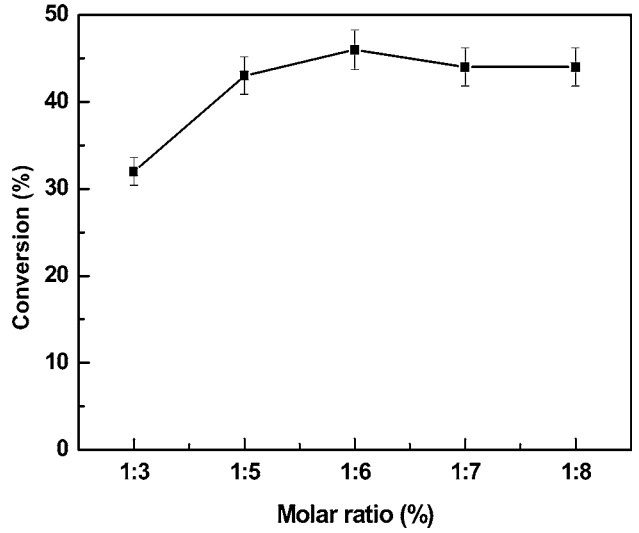

Figure 3. Effect of molar ratio of ascorbic acid to palmitic acid on the conversion of ascorbic acid to ascorbyl palmitate during surfactant-coated lipase mediated synthesis.

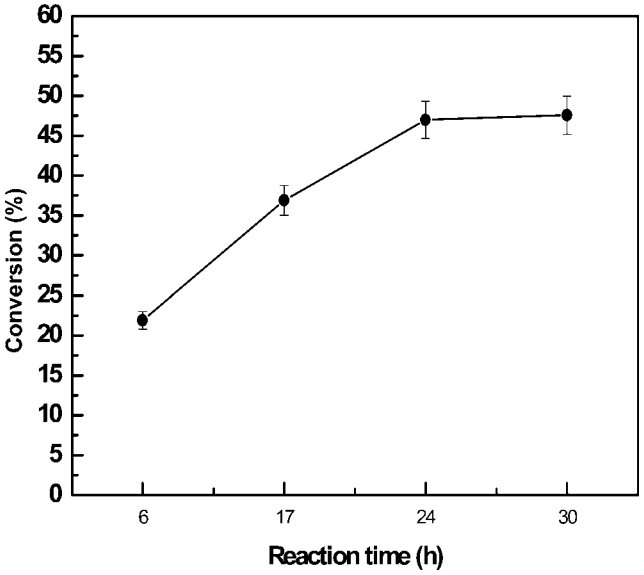

Figure 4. Time course of the formation of ascorbyl palmitate in *tert*-butyl alcohol catalyzed by the surfactant-coated lipase at 50 °C. The reaction medium was composed of 1:6 molar ratio of ascorbic acid to palmitic acid in 10 mL of *tert*-butyl alcohol, 0.3 g of surfactant-coated lipase, and 0.5 g of molecular sieve (4 Å).

were varied during the synthesis, and the results are shown in **Figure 3**. Highest conversion (47%) was obtained at a molar ratio of 1:6. The conversion obtained when free lipase was used as the biocatalyst in the synthesis of ascorbyl palmitate was only 6%, under the same conditions. This indicated that the crude *B. cepacia* lipase did not possess considerable catalytic activity in the synthesis of ascorbyl palmitate under the same conditions.

To determine the shortest time for achieving the highest conversion of ascorbic acid to ascorbyl palmitate, the time course of the reaction was monitored, and the result is shown in **Figure 4**. The production of ascorbyl palmitate increased rapidly up to 24 h of reaction. However, beyond this reaction time the conversion did not increase, which might be due to the reaction equilibrium. A maximum conversion of 47% was achieved in 24 h.

To conclude, this study confirmed the feasibility of synthesizing ascorbyl palmitate using surfactant-coated lipase as an acyl transfer catalyst. A few paper are available in the literature regarding the synthesis of ascorbyl palmitate employing commercial immobilized lipases. Using Novozym 435, Viklund et al. (*1*) obtained 86% conversion of ascorbyl palmitate after 50 h in *tert*-amyl alcohol. Similarly, using Novozym 435 in *tert*-amyl alcohol, Humeau et al. (*2*) obtained 56% conversion after 8 h. Although a comparably higher conversion could not be obtained by using surfactant-coated lipase, considering the cost of commercial immobilized lipases, the use of surfactant-coated lipase in the biosynthesis of ascorbyl palmitate would be advantageous. Improving the preparation method will certainly enhance further the catalytic activity of the surfactant-coated lipase for ascorbyl palmitate synthesis. Furthermore, coating of enzymes with biodegradable surfactants also avoids the need for toxic and harsh chemicals generally associated with the covalent coupling methods if the biocatalyst is prepared by immobilization to insoluble supports.

LITERATURE CITED

(1) Viklund, F.; Alander, J.; Hult, K. Antioxidant properties and enzymatic synthesis of ascorbyl fatty acid esters. *J. Am. Oil Chem. Soc.* **2003**, *80*, 795−799.

(2) Humeau, C.; Girardin, M.; Coulon, D.; Miclo, A. Synthesis of 6-*O*-palmitoyl L-ascorbic acid catalyzed by *Candida antartica* lipase. *Biotechnol. Lett.* **1995**, *17*, 1091−1094.

(3) Bradoo, S.; Saxena, R. K.; Gupta, R. High yields of ascorbyl palmitate by thermostable lipase-mediated esterification. *J. Am. Oil Chem. Soc.* **1999**, *76*, 1291−1295.

(4) Luhong, T.; Hao, Z.; Shehate, M. M.; Yunfei, S. A kinetic study of the synthesis of ascorbyl fatty acid esters catalyzed by immobilized lipase in organic media. *Biotechnol. Appl. Biochem.* **2000**, *32*, 35−39.

(5) Humeau, C.; Girardin, M.; Rovel, B.; Miclo, A. Enzymatic synthesis of fatty acid vitamin-C esters. *J. Mol. Catal. B: Enzym.* **1998**, *5*, 19−23.

(6) Yan, Y.; Bornscheurer, U. T.; Schmid, R. D. Lipase-catalyzed synthesis of vitamin-C fatty acid esters. *Biotechnol. Lett.* **1999**, *21*, 1051−1054.

(7) Goto, M.; Kameyama, H.; Goto, M.; Miyata, M.; Nakashio, F. Design of surfactants suitable for surfactant-coated enzymes as catalysts in organic media. *J. Chem. Eng. Jpn.* **1993**, *26*, 109−111.

(8) Goto, M.; Goto, M.; Kamiya, N.; Nakashio, F. Enzymatic interesterification of triglyceride with surfactant-coated lipase in organic media. *Biotechnol. Bioeng.* **1995**, *45*, 27−32.

(9) Goto, M.; Noda, S.; Kamiya, N.; Nakashio, F. Enzymatic resolution of racemic ibuprofen by surfactant-coated lipases in organic media. *Biotechnol. Lett.* **1996**, *18*, 839−844.

(10) Noda, S.; Kamiya, N.; Goto, M.; Nakashio, F. Enzymatic polymerization catalyzed by surfactant-coated lipases in organic media. *Biotechnol. Lett.* **1997**, *19*, 307−309.

(11) Huang, S. Y.; Chang, H. L.; Goto, M. Preparation of surfactant-coated lipase for the esterification of geraniol and acetic acid in organic solvents. *Enzyme Microb. Technol.* **1998**, *22*, 552−557.

(12) Basheer, S.; Cogan, U.; Nakajima, M. Esterification kinetics of long-chain fatty acids and fatty alcohols with a surfactant coated lipase in *n*-hexane. *J. Am. Oil Chem. Soc.* **1998**, *75*, 1785−1790.

(13) Hung, T. C.; Giridhar, R.; Chiou, S. H.; Wu, W. T. Binary immobilization of *Candida rugosa* lipase on chitosan. *J. Mol. Catal. B: Enzym.* **2003**, *26*, 69−78.

(14) Lotrakul, P.; Dharmsthiti, S. Lipase production by *Aeromonas sobria* LP004 in a medium containing whey and soybean meal. *World J. Microbiol. Biotechnol.* **1997**, *13*, 163−166.

(15) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(16) Tsuzuki, W.; Sasaki, T.; Suzuki, T. Effect of detergent attached to enzyme molecules on the activity of organic-solvent soluble lipase. *J. Chem. Soc., Perkin. Trans.* **1991**, *11*, 1851−1854.
(17) Okahata, Y.; Hatano, A.; Ijiro, K. Enhancing enantioselectivity of a lipid-coated lipase via imprinting methods for esterification in organic solvents. *Tetrahedron: Asymmetry* **1995**, *6*, 1311−1322.
(18) Zaka, A.; Klibanov, A. M. Enzyme-catalyzed processes in organic solvents. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 3192−3196.
(19) Chen, J. W.; Wu, W. T. Regeneration of immobilized *Candida antartica* lipase for transesterification. *J. Biosci. Bioeng.* **2003**, *95*, 466−469.

Received for review January 11, 2006. Revised manuscript received June 8, 2006. Accepted June 13, 2006.

JF060089D