Synthesis of Fatty Acid Esters by Recombinant *Staphylococcus epidermidis* Lipases in Aqueous Environment

Rey-Chang Chang,[‡] Shu-Jen Chou,[†] and Jei-Fu Shaw*,[†]

Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan 11529 and Department of Marine Biotechnology, National Kaohsiung Institute of Marine Technology, Number 142, Hai-Chuan Road, Nan-Tzu District Kaohsiung, Taiwan

Various flavor esters were obtained by using recombinant lipases from *Staphylococcus epidermidis* as a catalyst in an aqueous environment. These esters were enzymatically synthesized to overcome the problems associated with chemical processes. This study showed that the *S. epidermidis* lipases could catalyze ester synthesis from decyl alcohol and fatty acids of different chain length. The wild-type and mutant lipases (M419A and V649I) could efficiently catalyze the synthesis of decyl alcohol esters of unsaturated fatty acids. In contrast, the yield of decyl laurate was better by wild-type and mutant enzyme V649I, but mutant enzyme M419A only favored the synthesis of decyl myristate. The esterification of oleic acid and various carbon-chain-length alcohols from ethanol to hexadecanol increased up to decanol by wild-type and M419A mutant enzymes and reached an optimum for dodecanol by V649I mutant enzyme. The enzyme is potentially useful in food industries such as dairy product flavoring.

Keywords: *Enzymatic esterification; lipase; Staphylococcus epidermidis*

INTRODUCTION

Esters are common flavoring agents and are often employed in fruit-flavored products (e.g., beverages, candies, jellies, and jams), baked goods, wines, and dairy products (e.g., cultured butter, sour cream, yogurt, and cheese)(*1*). Esters have been isolated from all major food systems and are often expensive. Conventional esterification of flavor esters by chemical catalysts requires high temperature and leads to dark-colored products and undesired byproducts. Enzyme-catalyzed conversion is more efficient and selective (*2*). In recent years, there has been a strong demand for natural products, including natural flavors. This has created many opportunities for biocatalysis (use of enzymes) to compete with traditional synthetic chemistry for the production of flavors (*3*). Flavoring esters may be synthesized enzymatically utilizing inexpensive natural raw materials, such as fatty acids and alcohols (*4*). The use of lipolytic enzymes to catalyze the esterification reaction for producing flavor esters has been investigated by many workers (*5–7*). The esterification of alcohols and fatty acids catalyzed by lipolytic enzymes were performed in hydrophobic organic solvent or in aqueous–organic two-phase systems (*8–13*).

The *Staphylococcus epidermidis* lipase gene was first cloned by Farrell et al. (*14*). Recently, we have successfully expressed a highly active fragment in *Escherichia coli* and identified the residues essential to catalysis (*15*). The *S. epidermidis* lipase gene has been subjected to increased lipase activity and improved substrate specificity by site-directed mutagenesis. In comparison

with wild-type enzyme, the mutants (M419A and V649I) showed a 2.0 and 4.0-fold increase in the catalytic efficiency (k_{cat}/K_m), respectively (*15*). In the present work, we found that both the wild-type and mutant lipases (M419A and V649I) could efficiently synthesize various flavor esters in aqueous media. In the work, we have studied the parameters that affect the production of esters by *S. epidermidis* lipase in aqueous environments.

MATERIALS AND METHODS

Materials. Isopropyl thio-β-D-galactoside (IPTG), ampicillin, *p*-nitrophenyl butyrate, fast blue RR salt, α-naphthyl butyrate, oleic acid butyl ester, oleic acid lauryl ester, eicosapentaenoic acid (EPA), docosahexanoic acid (DHA), fatty acids, and alcohols were purchased from Sigma Co. Silica gel 60, hexane, 2-propanol, and acetone were purchased from Merck Chemical Co. Protein molecular weight markers were obtained from Novel Experimental Technology Co. Other chemicals were reagent grade. Double-distilled water was used throughout the experiment.

Expression of *S. epidermidis* lipases in *E.coli* host system. *E. coli* BL21(DE3) cells harboring the desired wild-type lipase gene on plasmid pET-20b(+) (*15*) were grown at 30 °C in LB/ampicillin to an optical (600 nm) density of 0.5. IPTG was added to the cultures (100 mL each) to a final concentration of 4 mM and the cells were harvested 3 h after the IPTG induction.

Protein Purification Procedures. For protein purification of the recombinant lipase, the cells [suspended in a buffer of 20 mM Tris-HCl (pH 7.9), 5 mM imidazole, 0.5 M NaCl, and 0.05% Tween 20] were broken on ice in a Microson ultrasonic cell disruptor (10 × 30 s at 50 w). After centrifugation (10000*g*), the supernatant fractions were loaded into NiSO$_4$-charged His-bind resins (Novagene Co.). After binding, the resins were washed with a buffer [20 mM Tris-HCl (pH 7.9), 60 mM imidazole, 0.5 M NaCl]. The lipase was eluted with another buffer [20 mM Tris-HCl (pH 7.9), 1 M imidazole, 0.5 M NaCl] and dialyzed against a 50 mM phosphate buffer (pH 6.0).

* To whom correspondence should be addressed. Telephone: 886-2-27899590, ext. 226 or 108. Fax: 886-2-27821605. E-mail: boplshaw@gate.sinica.edu.tw.
† Academia Sinica.
‡ National Kaohsiung Institute of Marine Technology.

Lipase Activity Staining by SDS−PAGE. The proteins in the supernatant fraction of broken cells were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (*16*). The separated proteins were stained with coomassie blue (*17*) and assayed for the esterase activity on gels (*18*) after the removal of SDS (*19*).

Determination of Protein Concentration. Protein concentrations were determined according to a dye-binding procedure (*20*), using the Bio-Rad protein assay kit system (Bio-Rad Laboratories, Hercules, CA).

Effect of *n*-Hexane Content on the Ester Synthesis. To study the effect of *n*-hexane content on the lipase-catalyzed synthesis, the cells harboring the lipase gene were broken on ice by an ultrasonic cell disruptor, and after centrifugation the supernatant fraction was crude enzyme and dialyzed against a 50 mM phosphate buffer (pH 6.0). Crude enzyme solution (1 mL) was mixed with 1 g of silica gel 60 and left for 30 min at 4 °C. The gel was precipitated by 8 mL of acetone (prechilled at 4 °C) and dried in a vacuum dryer. The immobilized lipase was transferred to a flask and vacuum-dried at ambient temperature.

Esterification Reaction. For standard reactions, the lipases solution (in 50 mM phosphate buffer pH 6.0) were added to a reaction mixture (1 mL) containing 50 mM fatty acids and 50 mM alcohols. The reaction mixture was carried out in screw-capped bottles (with 22-mm diameter) and incubated in an orbital shaker with a speed of 250 rpm at 33 °C.

Ester Analysis. At various time intervals, products were extracted in 1 mL of hexane, and 1.5 μL of the reaction mixture was withdrawn and analyzed by gas chromatography (Hitachi model 263-30; Hitachi, Tokyo, Japan). An DB-1 fused-silica capillary column of 15 m × 0.32 mm i.d. (J & W Corporation, Folsom, CA) was used. Nitrogen gas was the carrier gas at a flow rate of 1.2 mL/min. The injection port and flame ionization detector temperatures program was: 120 °C, raised at 20 °C/min to 250 °C, and 7 min hold. The product compositions were quantitated by an integrator with oleic acid lauryl ester or oleic acid butyl ester as internal standard. The conversion yield was calculated on the basis of the limited substrate.

Measurement of Lipase Activity. The lipase hydrolytic activities were measured by *p*-nitrophenyl butyrate as substrate. Detailed procedures are described by Rùa et al. (*21*). One unit was defined as the amount of enzyme that released 1μmol *p*-nitrophenol/min.

RESULTS AND DISCUSSION

The use of lipolytic enzymes to catalyze the esterification reaction for producing flavor esters in organic solvent has been investigated (*22, 23*) and the formation of ester may be strongly influenced by the water content in organic solvent (*24*). The immobilized *S. epidermidis* lipase was used for the enzymatic synthesis of octyl oleate in *n*-hexane with different water contents and the results are shown in Table 1. Surprisingly the yields of esterification were inversely proportional to the *n*-hexane content of the system. In the most successful attempts, it has been found that organic solvents nearly always exert deleterious effects on catalysis by both free and immobilized enzymes (*25*). This result reveals that *S. epidermidis* lipase became inactive in the *n*-hexane system, and an aqueous buffer system is essential for the catalytic activity of the *S. epidermidis* lipase.

To study the effect of esterification time, the yields of lauryl oleate as a function of esterification time are shown in Table 2. The yield of lauryl oleate catalyzed by *S. epidermidis* lipase reached a maximum at 12 h and then slightly decreased. For convenient standard experiments, we carried out the esterification reaction for 10 h.

Table 1. Effect of *n*-hexane Content on the Synthesis of Octyl Oleate by the Recombinant *S. epidermidis* Lipase[a]

n-hexane content (%)	yield (%)[b]
0	46.5
10	26.5
20	11.0
30	4.3
40	3.5
50	3.0
60	2.7
70	1.9
80	1.2
90	0
100	0

[a] Experimental conditions: lipase was immobilized on silica gel 60 as described under Materials and Methods. Reaction conditions: 0.15 g immobilized wild-type lipase (21500 units), 50 mM oleic acid, and 50 mM octanol in 1 mL of mixture solvent of hexane and 50 mM phosphate buffer (pH 6.0), shaken at 250 rpm for 10 h at 33 °C. Enzyme unit was measured by the hydrolysis of *p*-nitrophenyl butyrate as substrate. One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of *p*-nitrophenol/min. [b] The percentage of yield was defined as the molar ratio of ester to original reactant.

Table 2. Effect of Reaction Time on Synthesis of Lauryl Oleate by the Recombinant *S. epidermidis* Lipase[a]

reaction time (h)	yield (%)[b]
3	11.1
6	47.7
9	63.1
12	68.8
15	67.3
18	64.4
22	65.8
24	63.1
48	64.2
128	53.1

[a] The enzyme (19200 units) was added to a reaction mixture (1 mL) containing 50 mM oleic acid and 50 mM lauryl alcohol in 50 mM phosphate buffer (pH 6.0) at 33 °C. The reaction times were 3, 6, 9, 12, 15, 18, 22, 24, 48, and 128 h, respectively. [b] The percentage of yield was defined as the molar ratio of ester to original reactant.

Table 3. Effect of Chain Length of Primary Acids on the Synthesis of Decyl Esters by the Recombinant *S. epidermidis* Lipases[a]

	yield (%)[b]		
primary acid	wild type	M419A	V649I
acetic acid	0	0	0
butyric acid	0	0	0
hexanoic acid	0.85	0	1.55
octanoic acid	23.1	11.97	23.1
decanoic acid	24.79	0.50	25.85
lauric acid	59.58	16.76	51.41
myristic acid	31.69	40.85	17.04
palmitic acid	13.94	12.82	13.17
stearic acid	12.11	11.69	12.11
arachidonic acid	2.11	12.68	0

[a] The enzyme (16500 units) was added to a reaction mixture (1 mL) containing 50 mM acid and 50 mM lauryl alcohol in 50 mM phosphate buffer (pH 6.0) at 33 °C for 10 h. [b] The percentage of yield was defined as the molar ratio of ester to original reactant.

The yield of esters was affected by acyl donors. As shown in Table 3, wild-type and V649I mutant enzymes showed higher esterification yield with the medium-chain fatty acids (from C8 to C14) and reached an optimum for lauric acid. In comparison with the wild-type enzyme, the M419A mutant enzyme revealed slightly improved esterification yield with further lengthening of the carbon chain of acids and reached an

Esters Synthesis by Recombinant *Staphylococcus* Lipase

J. Agric. Food Chem., Vol. 49, No. 5, 2001 **2621**

Table 4. Effect of Acids Structure on the Synthesis of Decyl Esters by the *S. epidermidis* Lipases[a]

	yield (%)[b]		
acid	wild type	M419A	V649I
stearic acid	12.11	11.69	12.11
oleic acid	65.32	62.01	58.36
linoleic acid	43.54	10.30	38.26
EPA[c]	6.82	3.25	6.59
DHA[c]	49.81	0.60	25.35
benzoic acid	0	0	0
cyclohexane-carboxylic acid	0	0	0.12

[a] The enzyme (16500 units) was added to a reaction mixture (1 mL) containing 50mM fatty acids and 50 mM decyl alcohol in 50 mM phosphate buffer (pH 6.0) at 33 °C for 10 h. [b] The percentage of yield was defined as the molar ratio of ester to original reactants. [c] EPA, eicosapentanenoic acid; DHA, docosahexanoic acid.

optimum for myristic acid. Our site-directed mutagenesis results indicate that Met419 might play a dominant role in the acid-binding and Val649 might not affect the acid-binding. These are consistent with the previous hypothesis that suggests the residues behind the serine and histidine of the catalytic triad might be involved in acid-binding and alcohol-binding, respectively (*26*). M419A may be related mainly to the change in the molecular weight of amino acid side chains that are essential for the catalytic activity of the enzyme (the molecular weight of methionine and alanine are 149 and 89, respectively, and the hydropathy indexes are 1.9 and 1.8, respectively). In our previous study, the mutants (M419A and V649I) compared with the wild type showed a 2.0 and 4.0-fold increase in the catalytic efficiency (k_{cat}/K_m) of ester hydrolysis (*15*), but the wild type and V649I had similar catalytic efficiency in the ester synthesis. Therefore, there is little correlation between the synthesis and hydrolytic activities, as reported by Wu et al. (*27*). Similar studies in other staphylococcal lipases were observed by Talon et al. (*28*). They produced ethyl esters from hexanoic and oleic acids in *n*-hexane by *S. warneri* and *S. xylosus* lipases and the esterification yields reached an optimum for the decanoic acid. Under their conditions, the esterification yield of oleic acid was only half that of decanoic acid (*28*).

To study the effect of acyl donors, the lipase-catalyzed esterification reactions of decyl alcohol with various acids were investigated. As shown in Table 4, the yields of decyl esters with the primary unsaturated acids (oleic acid, linoleic acid, and DHA, but not EPA) were much higher than those with the corresponding saturated primary acid and reached an optimum for oleic acid in reactions catalyzed by the wild-type and V649I mutant enzymes. Conversely, in comparison with the wild-type, the M419A mutant enzyme showed a dramatic decrease in the conversion yield of DHA ester. These results suggested that M419 is an essential element for acid-binding. This is consistent with the previous suggestion for M419A which causes a slight conformational change around the acid-binding and tends to decrease in the substrate specificity for the esterification of DHA ester. On the other hand, the *S. epidermidis* lipases (wild-type, M419A, and V649I) could catalyze ester synthesis from decyl alcohol and fatty acids with a primary straight carbon chain, but they could not esterify carboxylic acid having both aliphatic and aromatic cyclic carbon chains such as cyclohexane-carboxylic acid and benzoic acid (Table 4).

The yields of esters were affected by different alcohols. As shown in Table 5, the yield of decanoyl oleate was

Table 5. Effect of Chain Length of Primary Alcohols on Esterification with Oleic Acid by the Recombinant *S. epidermidis* Lipases[a]

	yield (%)[b]		
primary alcohol	wild type	M419A	V649I
ethanol	0	0	0
butanol	11.13	3.1	21.29
hexanol	3.39	3.39	65.81
octanol	14.0	8.71	57.29
decanol	64.35	60.19	57.1
dodecanol	34.35	34.35	84.19
tetradecahol	18.39	34.35	39.68
hexadecanol	21.19	24.19	25.35

[a] The enzyme (18600 units) was added to a reaction mixture (1 mL) containing 50 mM oleic acid and 50 mM primary alcohols at 33 °C for 10 h. [b] The percentage of yield was defined as the molar ratio of ester to original reactants.

Table 6. Effect of Alcohols Structure on Esterification with Oleic Acid by the Recombinant *S. epidermidis* Lipases[a]

	yield (%)[b]		
alcohol	wild type	M419A	V649I
primary alcohols			
1-decanol	64.35	60.19	57.1
geraniol	38.56	14.87	75.24
oleyl alcohol	37.05	24.35	43.28
secondary alcohols			
2-butanol	0	0	0
cyclohexanol	1.69	0	1.03
tertiary alcohol			
tertiary butanol	0	0	0

[a] The enzyme (18600 units) was added to a reaction mixture (1 mL) containing 50 mM oleic acid and 50 mM alcohols at 33 °C for 10 h. [b] The percentage of yield was defined as the molar ratio of ester to original reactants.

much better by wild-type and M419A mutant enzymes. For V649I mutant enzyme, the yields of butanol, hexanol, octanol, and dodecanol were much higher than those of the wild-type and M419A mutant enzymes. In comparison with the wild-type enzyme, the M419A mutant enzyme showed similar tendencies, and the V649I mutant enzyme had much broader alcohol selectivity for ester synthesis. These results indicated that V649 might be an essential residue for alcohol-binding in ester synthetic activity. It corresponds with the previous proposal for the role of the residues behind the histidine of the catalytic triad, that may be involved in alcohol-binding (*26*). To study the effect of alcohols of different carbon chains on the ester synthesis activity, oleic acid was reacted with primary alcohols (1-decanol, geraniol, and oleyl alcohol), secondary alcohols (2-butanol and cyclohexanol), and a tertiary alcohol (tertiary butanol) (Table 6). The esterification with primary alcohols had much higher yield than those of secondary and tertiary alcohols by wild-type and mutant enzymes.

The recombinant lipases of *S. epidermidis* are particularly interesting because they allow catalysis of ester synthesis without organic solvents. This system presents the following advantages: (i) avoiding the problem of toxicity and flammability of organic solvents; and (ii) simplification of product purification conditions. Considering the specificity of the enzyme, it could be used to produce medium-chain esters, geranyl esters, and unsaturated esters. The enzyme could be further engineered for the synthesis of short-chain esters and some useful fatty acids. The improvement of substrate speci-

ficity and catalytic efficiency by protein engineering are under investigations.

LITERATURE CITED

(1) Kim, J.; Altreuter, D. H.; Clark, D. S.; Dordick, J. S. Rapid Synthesis of fatty acid esters for use as potential food flavors. *J. Am. Oil Chem. Soc.* **1998**, *75*, 1109−1113.

(2) Shaw, J. F.; Lo, S. Production of propylene glycol fatty acid monoesters by lipase-catalyzed reactions in organic solvents. *J. Am. Oil Chem. Soc.* **1994**, *71*, 715−719.

(3) Whitehead, I. M. Challenges to biocatalysis from flavor chemistry. *Food Technol.* **1998**, *52*, 40−46.

(4) Manjon, A.; Iborra, J. L.; Arocas, A. Short-chain flavor ester synthesis by immobilized lipase in organic media. *Biotechnol. Lett.* **1991**, *13*, 339−344.

(5) Karra-Chaabouni, M.; Pulvin, S.; Touraud, D.; Thomas, D. Parameters affecting the synthesis of geranyl butyrate by esterase 30,000 from *Mucor miehei. J. Am. Oil Chem. Soc.* **1998**, *75*, 1201−1206.

(6) Welsh, F. W.; Williams, R. E. Lipase mediated production of flavor and fragrance esters from fusel oil. *J. Food Sci.* **1989**, *54*, 1565−1568.

(7) Langrand, G.; Triantaphylides, C.; Baratti, J. Lipase catalysed formation of flavor esters. *Biotechnol. Lett.* **1988**, *10*, 549−554.

(8) Carta, G.; Gainer, J. L.; Benton, A. H. Enzymatic synthesis of ester using an immobilized lipase. *Biotechnol. Bioeng.* **1991**, *37*, 1004−1009.

(9) Lima, F. V.; Pyle, D. L.; Asenjo, J. A. Factors affecting the esterification of lauric acid using an immobilized biocatalyst: Enzyme characterization and studies in a well-mixed reactor. *Biotechnol. Bioeng.* **1995**, *46*, 69−79.

(10) Shaw, J. F.; Wang, D. L.; Wang, Y. J. Lipase-catalyzed ethanolysis and 2-propanolysis of triglycerides with long-chain fatty acids. *Enzyme Microb. Technol.* **1991**, *13*, 544−546.

(11) Monot, F.; Borzeix, F.; Bardin, M.; Vandecasteele, J. P. Enzymatic esterification in organic media: Role of water and organic solvent in kinetics and yield of butyl butyrate synthesis. *Appl. Microbiol. Biotechnol.* **1991**, *35*, 759−765.

(12) Eggers, D. K.; Blanch, H. W.; Prausnitz, J, M. Extractive catalysis: Solvent effects on equilibria of enzymatic reactions in two-phase systems. *Enzyme Microb. Technol.* **1989**, *11*, 84−89.

(13) Golberg, M.; Legoy, M. D. Water activity as a key parameter of synthesis reactions: The example of lipase in biphasic (liquid/solid) media. *Enzyme Microb. Technol.* **1990**, *12*, 976−981.

(14) Farrell, A. M.; Foster, T. J.; Holland, K. T. Molecular analysis and expression of the lipase of *Staphylococcus epidermidis. J. Gen. Microbiol.* **1993**, *139*, 267−277.

(15) Chang, R. C.; Chou, S. J.; Shaw, J. F. Site-directed mutagenesis of a highly active *Staphylococcus epidermidis* lipase fragment identifies residues essential for catalysis. *J. Am. Oil Chem. Soc.* **2000,** *77*, 1021−1025.

(16) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(17) Bollag, D. M.; Edelstein, S. J. *Protein Methods*; Wiley-Liss, Inc.: New York, 1991; p 155.

(18) Thanksley, S. D.; Orton, T. T. Isozymes in Plant Genetics and Breeding, Part A. Elsevier Science Publishers: New York, 1983; pp 500−501

(19) Blank, A.; Sugiyama, R. H.; Dekker, C. A. Activity staining of nucleolytic enzymes after sodium dodecyl sulfate−polyacrylamide gel electrophoresis: Use of aqueous 2-propanol to remove detergent from gels. *Anal. Biochem.* **1982**, *120*, 267−275.

(20) 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.

(21) Rúa, M. L.; Diaz-Maurino, T.; Fernandez, V. M.; Otero, C.; Ballesteros, A. Purification and characterization of two distinct lipases from *Candida cyclindracea. Biochim. Biophys. Acta* **1993**, *1156*, 181−189.

(22) Welsh, F. W.; Williams, R. E. Lipase-mediated production of ethylbutyrate and butylbutyrate in nonaqueous media. *Enzyme Microb. Technol.* **1990**, *12*, 243−248.

(23) Oguntimein, G. B.; Anderson, W. A.; Moo-Young, M. Synthesis of geraniol esters in a solvent-free system catalyzed by *Candida antarctica* lipase. *Biotechnol. Lett.* **1995**, *17*, 77−82.

(24) Claon, P. A.; Akoh, C. C. Enzymatic synthesis of geraniol and citronellol esters by directed esterification in *n*-hexane*. Biotechnol. Lett.* **1993**, *15*, 1211−1215.

(25) Gatfield, I. L. The enzymatic synthesis of esters in nonaqueous systems. *Lebensm.-Wiss. Technol.* **1986**, *19*, 87−88.

(26) Kazlauskas, R. T. Elucidating structure-mechanism relationships in lipases: prospects for predicting and engineering catalytic properties. *Trends Biotechnol.* **1994**, *12*, 464−472.

(27) Wu, X. Y.; Jääskeläien, S.; Linko, Y. Y. An investigation of crude lipases for hydrolysis, esterification and trans-esterification. *Enzyme Microb. Technol.* **1996**, *19*, 226−231.

(28) Talon, R.; Montel, M. C.; Berdague, J. L. Production of flavor esters by lipases of *Staphylococcus warneri* and *Staphylococcus xylosus. Enzyme Microbial. Technol.* **1996**, *19*, 620−622.

(29) Parida, S.; Dordick, J. S. Tailoring lipase specificity by solvent and substrate chemistries. *J. Org. Chem.* **1993**, *58*, 3238−3244.