Synthesis of Medium-Chain Glycerides by Lipase in Organic Solvent

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ABSTRACT: Using commercial lipases from various microbial origins, medium-chain glycerides, such as mono-, di-, and tricaprin, were synthesized in isooctane from glycerol and capric acid. The enzyme reaction was performed with 0.35 M capric acid, 0.025 M glycerol, and 0.46 g silica gel to remove water in 5 mL of isooctane with 30 mg lyophilized lipase. Of the 21 kinds of lipases, 11 showed good synthetic activities. Lipases from *Pseudomonas aeruginosa* (Lipase PS), *Rhizomucor miehei* lipase and *Chromobacterium viscosum* lipase (Lipase CV) showed high activities for the production of tricaprin, while lipase OF-360 (from *Candida rugosa*) and lipase D *(Rhizopus delemar)* were good for dicaprin production. Lipases CC and MY from *C. rugosa (C. cylindracea)* also showed high activities for dicaprin and tricaprin. Some lipases, especially lipase PS, had high thermal stability over 60°C. The optimal lyophilization pH to dehydrate the lipase coincides with the optimal buffer solution pH for hydrolysis.

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KEY WORDS: Lipase, medium-chain glyceride, synthetic reaction.

The medium-chain glycerides, mono-, di, and tri-glycerides, having fatty acids composed of medium-chain fatty acids, such as caprylic and capric acids, have been used as a component for infant feeding formula and as a nutritional supplement for patients suffering from malabsorption caused by disease or intestinal resection (1,2). While long-chain triglycerides require the presence of pancreatic-biliary secretion and an intact intestinal lymphatic system for absorption, medium-chain triglycerides can penetrate intestinal cells directly and can be absorbed directly into the portal venous system more rapidly (3).

The commercial manufacturing process of such medium-chain glycerides is the direct synthesis from medium-chain fatty acids and glycerol at high temperature and high pressure, followed by alkali washing, steam refining, molecular distillation, and ultrafiltration for purifying the product (4). However, these conventional chemical procedures are very expensive due to the extreme conditions; thus enzymatic synthesis was focused on the esterification of this medium-chain glyceride from fatty acids and glycerol using lipases (5,6).

Generally it is not common to carry out the enzyme-catalyzed synthesis in water, because water in the solvent drives the reaction equilibrium in the direction of hydrolysis (6,7). This is the reason why most of the enzymes, such as esterase, protease, and amylase, are known for their hydrolytic activities, even though these enzymes can catalyze the reverse synthetic reaction. Synthetic reaction of the enzymes for any biomolecule rarely takes place without eliminating the water in the reaction media *in vitro* (7).

Thus we applied the nonaqueous organic solvent system (6) to the esterification of glycerol with fatty acid using lipases (8). A nonaqueous organic solvent system has an advantage over an aqueous system for synthesizing the glycerides because water can be eliminated (6,9). The objective of this paper was to screen commercial lipases and organic solvents for optimum synthesis of the individual medium-chain glycerides, and to study their lipase reaction properties in organic solvents.

MATERIALS AND METHODS

Lipases. Lipase CES (*Pseudomonas* sp.), Lipase AY *(Candida rugosa),* Lipase AP-10 *(Aspergillus niger),* Lipase GT-20 *(Penicillium cyclopium),* Lipase GC *Geotrichurn candidum),* Lipase AP *(A. niger),* Lipase PS *(Pseudomonas aeruginosa),* Lipase L *(C. lipolytica),* Lipase F-AP 15 *(Rhizopus javanicus),* Lipase R *(Penicillium roqueforti),* Lipase D *(R. delemar),* Lipase CE *(Humicola lanuginosa),* and Lipase G *(P. cyclopium)* were supplied from Amano Enzyme Co. (Nagoya, Japan). Two lipases from *C. cylindracea* and *Pancreatic lipase* were purchased from Sigma Chemical Co. (St. Louis, MO). Lipase from *Rhizomucor miehei* and Lipozyme IM-20 (immobilized *R. miehei* lipase) were obtained from Novo Enzyme Co. (Bagsvaerd, Denmark). Lipase MY and Lipase OF-360 *(C. rugosa)* were from Meito Sankyo (Osaka, Japan) and Lipase CV *Chromobacterium viscosum)* was from Toyo Jozo (Shizuoka, Japan).

Chemicals. Lipids standards, such as capric acid, monocaproylglycerol (monocaprin), dicaproylglycerol (dicaprin), and tricaproylglycerol (tricaprin), to identify and construct the calibration curves were purchased from Sigma. Glycerol

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(molecular biology certified grade) used as a substrate was purchased from Kodak International Biotechnologies Inc. (New Haven, CT). Water in glycerol, determined by Karl Fisher's method using the Dosimat 665 (Metrohm Co., Swiss), was 0.06% (w/w). Isooctane (2,2,4-trimethylpentane) was purchased from Aldrich Chemical Co. (Milwaukee, WI), and acetonitrile and isoproyl alcohol (2-propanol) were of high-performance liquid chromatography (HPLC) pure grade. Wakogel C-300 (230–400 mesh; Wako Chemical Co., Osaka, Japan) was used as a silica gel. All other chemicals and solvents were of analytical grade.

Enzyme reaction. Lipase (500 mg) was dissolved in 500 mL of 0.01 M phosphate buffer solution (pH 7.0) and was lyophilized (see pH memory theory; 10). Typical enzymatic synthesis of medium-chain glyceride was performed by adding 30 mg of the lyophilized lipase to 5 mL of isooctane (11) in the presence of 0.35 M of capric acid and 0.025 M of glycerol (8,12). Water in isooctane was removed by adding molecular sieve just before use (13). Approximately 0.46 g Wakogel was added to the mixture (5 mL solvent) to remove the water produced during the reaction (7). Reaction vials were previously silanized with 5% dichlorodimethylsilane in toluene (8,12) to avoid binding the glycerol to the vial by reducing the surface tension of glycerol against the glass wall (12). After a 10-s sonication, the reaction vials were placed on a submerged magnetic stirrer (500 rpm, VarioMag Telemodule 20P; HTP Labortechnik, Munich, Germany), and the temperature was kept at 25°C. Periodically, aliquots were withdrawn, and the amounts of capric acid, mono-, di-, and tricaprin were assayed by HPLC.

Lipase activity assay. The lipase activities were determined by measuring the amounts of mono-, di, and tricaprin produced by analytical HPLC (Waters, Milford, MA). Reversed-phase Novapak C_{18} column (8 mm i.d. × 10 cm, 4 μm) was used, and the mobile phase was acetonitrile/2-propanol/acetic acid (15:15:1, vol/vol/vol) (5). The flow rate was isocratically controlled at 0.5 mL/min. The peaks were detected by RI detector (Waters Model 410 Differential Refractometer) with the areas integrated by Waters Data Module (Model 745). The amount of each product synthesized was calculated from standard curves that were constructed from authentic samples of monocaprin, dicaprin, tricaprin, and capric acid ranging from 10 to 250 mM. The injection volume was 4 μL.

Effects of pH and temperature on synthetic activities. Lipase from *Aspergillus niger* was lyophilized at different pH. Buffers used were citrate buffer for pH 3, 4, and 5; succinate buffer for pH 4, 5, and 5.5; phosphate buffer for pH 6, 6.5, 7, and 8; and histidine buffer for pH 9. The buffer concentration was 0.05 M. After lyophilization, enzymes were washed with acetonitrile to remove the salt on the glass filter. The amount of enzyme loaded was adjusted to 30 mg of protein concentration as determined by Lowry method (14). After reacting for 10 h, the amount of monocaprin, dicaprin, and tricaprin produced were determined by HPLC.

The effect of temperature on the synthetic activity using Lipase PS and *R. miehei* lipase was investigated at various temperatures: 20, 30, 40, 50, 60, 70, and 80°C during a 10-h reaction period. Isooctane was used as the reaction medium, and the reaction vessel was tightly sealed to avoid evaporation.

RESULTS AND DISCUSSION

The standard curves of monocaprin, dicaprin, tricaprin, and capric acid constructed showed good linearity between peak areas and sample concentrations from 20 to 250 mM (data not shown). To select suitable enzymes for synthesizing the medium-chain glycerides, 20 lipases were tested for their synthetic activities in isooctane. As shown in Table 1, Lipase PS, Lipase D, Lipase CC, Lipase MY, Lipase OF-360, Lipase CV, Lipase AP, and *R. miehei* lipase showed relatively good activity for synthesizing the monoglycerides, diglycerides, and triglycerides of medium-chain fatty acids. However, Lipase R, Lipase CES, Lipase L, and pancreatic lipase showed no activity. Lipase CE, Lipase G, Lipase GC, and Lipase GT-20 showed poor activity.

These results are quite different from the reported data for the esterification of some acid (13) and alkyl esterification of some fatty acids (15). In methylesterification of trifluoromandelic acid, lipases from *H. lanuginosa* (originally the same as Lipase CE), *C. cylindracea* (same as Lipase CC), *Aspergillus* sp. (similar to Lipase AP), *Chr. viscosum,* and lipoprotein lipase showed good activity (13), whereas Lipase GC *(G. candidum),* lipases from *R. javanicus* (same origin as Lipase F-AP15), *R. delemar* (same as Lipase D), and *P. cyclopium* (similar to Lipase GT-20) showed poor activity (13). Cloan and Akoh (15) reported that Lipase SP-435 (immobilized lipase from *C. antarctica*) and IM-60 (immobilized lipase from *Mucor miehei*) exhibited the best performance for synthesis of terpene esters by transesterification of acids, such as acetic acid and butyric acid, while Lipase PS and Lipase AK (*Pseudomonas* sp.) and lipase from *C. cylindracea* showed poor activity. Interestingly, SP-435 failed to accommodate tricaprin as an acyl donor, although SP-435 was the best to attack the acetic acid and butyric acid.

From our results and that of other results (13,15,16), it can be concluded that fatty or aliphatic acid esterification by lipases are dependent on the nature of compounds used (glycerol, methanol, terpene, or glucose) and vary from one enzyme to another. Hydrolytic activities of some lipases are dependent on the fatty acid chainlength (17).

The lipases that had good synthetic activities in organic solvent can be grouped into three classes according to the synthetic patterns (Table 1). Group I consists of Lipase CC *(C. cylindracea),* Lipase OF-360 (originated from *C. rugosa*), Lipase AY *(C. rugosa),* and Lipase MY *(C. rugosa)* and produces a maximum amount of dicaprin with a continuous accumulation throughout the reaction. Monocaprin was rapidly produced within 2 h, but with a slight decrease later in the reaction. Group II consists of Lipase PS *(P. aeruginosa),* Lipase CV *(Chr. viscosum),* and *R. miehei* lipase that showed good activity for the production of tricaprin. In the first 2 h of reac-

TABLE 1
Synthetic Activities of the Medium-Chain Glycerides for the Various Lipases[a]

	Products					
	Monocaprin		Dicaprin		Tricaprin	
	Reaction time (h)					
Lipase	2	18	2	18	2	18
F-AP15 *(Rhizopus javanicus)*	+	++	++	+++++	+	++
R *(Penicillium roquerforti)*	—	—	—	—	—	—
PS *Pseudomonas aeruginosa)*	+	+	+++	+++++	+	+++++
D *(R. delemar)*	+	+	+	++++	+	+
Pancreatic lipase	—	—	—	—	—	—
CE *(Humicola lanuginosa)*	trace	+	trace	+	trace	+
G. *(P. sp.)*	+	++	+	++	—	—
G *(P. javanicus)*	+	++	+	++	trace	+
CC *(Candida cylindracea)*	++	+	+++	++++++	+	+++
Lipase MY *(C. rugosa)*	+	++++	++	++++++	++	+++
Rhizomucor miehei	++	+	+++++	++++	++	++++++
OF-360 *(C. rugosa)*	++++	++++	+++	++++++	+	+
CV *(Chromobacterium viscosum)*	++	+	++++	+++	trace	+++++
CES *(Pseudomonas sp.)*	—	—	—	—	+	—
L *(C. lipolytica)*	—	—	—	—	—	—
AY *(C. rugosa)*	+	++	+++	+++	+	++
GC *(Geotrichum candidum)*	+	+	+	++	+	+++
AP *(Aspergillus niger)*	+	++	+	+++++	+	+++
M-AP10 *(A. niger)*	trace	+	+	++++	trace	++
GT-20 *(P. cyclopium)*	trace	+	trace	+	trace	trace

[a]+, 0–10000; ++, 10000–20000; +++, 20000–35000; ++++, 35000–50000; +++++, 50000–65000; ++++++, over 65000. The numbers are peak areas given as the integral area computed by the high-performance liquid chromatography integrator (Waters 745B; Waters, Milford, MA).

tion, rapid production of monocaprin and dicaprin occurred which decreased as the reaction progressed. The maximum amount of tricaprin was produced after the reaction proceeded for 18 h. Group III is represented by Lipase AP *(A. niger)*, Lipase F-AP15 *(R. javanicus)*, and Lipase D *(R. delemar)*, which produced the maximum amount of dicaprin, and also produced tricaprin and monocaprin. The amount of any product was not decreased during the reaction.

The lipases belonging to Group I are known as nonspecific lipases in hydrolysis reactions (17). Although Group II and Group III lipases are all the same 1,3-hydrolysis-specific lipases in hydrolysis reactions (17,18), they gave different profiles in synthesizing medium-chain glycerides in organic solvents. Group II lipases were good for the production of tricaprin, while Group III were good for dicaprin production.

To select the best solvent for enzyme-catalyzed esterification, we applied five water-immiscible solvents, including isooctane, using *C. rugosa* lipase (Lipase OF-360). Table 2 shows that hexane was the best and isooctane was the second best for synthesis of medium-chain glyceride. In hexane, 81% of capric acid was esterified to monocaprin, dicaprin, and tricaprin. The conversion yield in isooctane was 73%. When the same experiments were carried out with the lipases from *R. javanicus* (F-AP15) and *R. miehei*, hexane was the best solvent (data not shown). These results are different from the data of a two-phase system for fat hydrolysis. In the two-phase system, isooctane was the best because lipase was more stable in isooctane than in any other solvent (11). The synthetic profiles of lipases in the various solvents did not differ to any great extent (data not shown) except hexane, which showed a slightly different synthetic profile from that of the other solvents, especially with *C. rugosa* lipase (Table 2). Figure 1 shows the time course of synthesis of monocaprin, di-

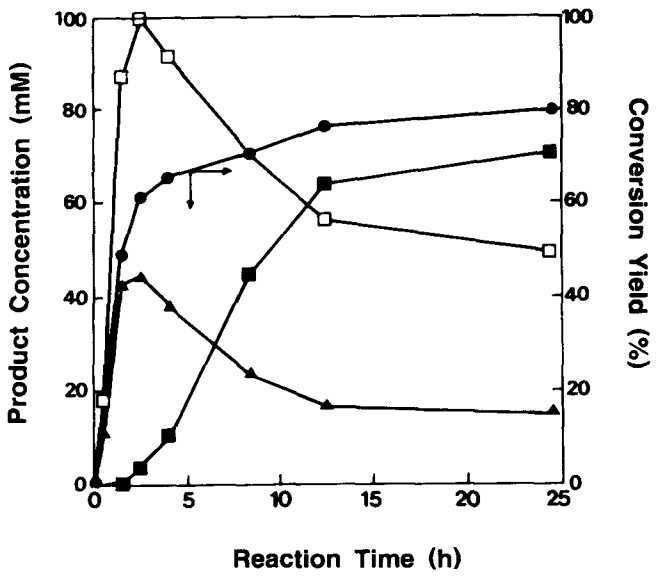

FIG. 1. Synthesis profile of medium-chain glyceride by the lipase from *Rhizomucor miehei* in hexane and conversion yield: □, monocaprin; ▲, dicaprin; ■, tricaprin; ●, conversion yield (%).

TABLE 2
Amounts of Monocaprin, Dicaprin, and Tricaprin Produced by Lipase from *Candida rugosa* in Various Solvents and Conversion Yield

Solvent	Reaction time (h)	Concentration (mM)[a]			Conversion yield (%)[b]
		Monocaprin	Dicaprin	Tricaprin	
Cyclohexane	1.5	31.81	30.08	2.81	19.68
	3	26.46	64.71	9.54	48.58
	5	22.84	72.67	15.97	58.96
	8	21.93	74.98	23.22	63.76
	14.5	19.42	67.95	31.75	68.30
	24	18.14	63.39	38.13	73.59
Heptane	1.5	37.19	24.48	1.85	21.34
	3	36.38	48.83	4.83	38.81
	5	34.84	61.95	6.92	46.43
	8	36.15	71.59	7.97	52.74
	14.5	32.49	83.04	10.41	58.60
	24	28.74	78.50	12.56	67.61
Octane	1.5	35.46	30.68	1.31	25.96
	3	36.22	53.95	3.62	37.30
	5	31.75	65.21	3.99	47.59
	8	30.80	77.28	6.93	54.69
	14.5	27.36	81.05	11.47	62.03
	24	25.06	75.59	18.34	65.89
Hexane	1.5	19.04	51.17	10.71	35.91[c]
	3	12.82	43.19	32.90	54.07[c]
	5	9.59	33.57	43.93	65.87[c]
	8	9.19	34.06	52.22	69.47[c]
	14.5	8.31	32.11	50.59	75.64[c]
	24	7.29	29.80	47.62	81.64[c]
Isooctane	1.5	39.47	39.88	1.36	34.88
	3	38.04	62.17	3.63	46.23
	5	34.57	74.96	5.87	54.33
	8	32.49	81.82	9.65	61.85
	14.5	27.85	75.82	17.70	68.71
	24	24.43	72.14	27.67	73.04

[a]Average product concentration from duplicate experiments.
[b]Conversion yield (%) = [MC × 1) + (DC × 2) + (TC × 3)]/[CA (350 mM)] × 100, where MC, DC, TC, and CA are the abbreviations of monocaprin, dicaprin, tricaprin, and capric acid, respectively.
[c]Calibrated conversion yield with the evaporated volume considered.

caprin, and tricaprin from capric acid by *R. miehei* lipase in hexane. In the first 3 h of the reaction, almost 60% of capric acid was converted to tricaprin, dicaprin, and monocaprin. After 3 h of reaction, the monocaprin and dicaprin produced were converted to tricaprin and the yield increased slightly to 80%. In the following experiments, however, we used isooctane instead of hexane with tight sealing, because hexane evaporated easily. In fact, 1% (vol/vol) of hexane evaporated every 1 h, even when the reaction bottle was sealed with Teflon stopper. Thus it was very difficult to prevent the evaporation of hexane during the reaction, especially during sampling for HPLC injection.

Lipase AP *(A. niger)* had no esterification activities for capric acid when the lipase was lyophilized at pH 3, 4, 5, 8, and 9. Figure 2 shows the effect of pH during enzyme lyophilization on the synthesis activity from pH 5.5 to 7.5 at 25°C. Lipase AP was used for this study because it belonged to Group III lipases that produced dicaprin, tricaprin, and

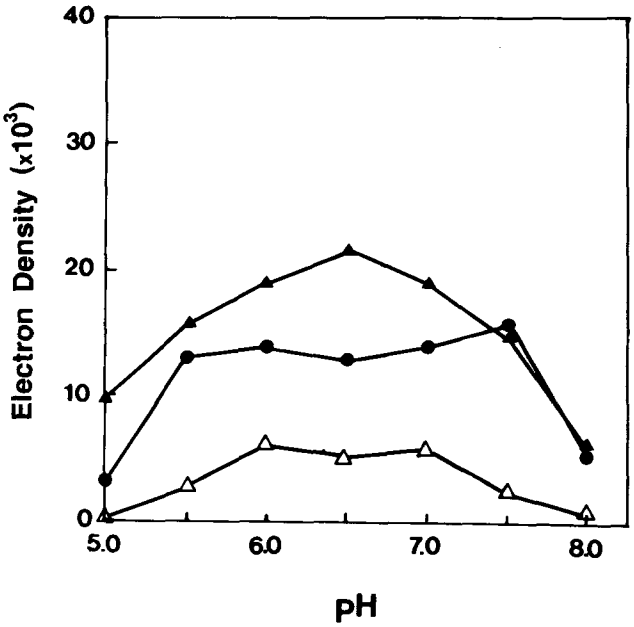

FIG. 2. Effect of lyophilization pH on the synthesis of medium-chain glyceride in isooctane by lipase from *Aspergilus niger*. △, tricaprin; ●, monocaprin; ▲, dicaprin.

monocaprin without decreasing throughout the reaction. The activity was determined after a reaction period of 10 h. When lyophilization was done at pH 6.5, all products, such as dicaprin, monocaprin, and tricaprin, were synthesized at maximal levels. Usually, the optimal pH of the lipase for hydrolytic activity in an aqueous solvent ranged from pH 6.0 to 7.0 (9,10,19). This result showed that the optimum pH for lyophilization of lipase for esterification in an organic solvent coincides with the optimum pH of hydrolytic activities in an aqueous system (10) or a two-phase system (19). The enzymatic reaction in isooctane very much depends on the pH of the last aqueous solution to which the enzyme was exposed, because the conformation of lipase in water-immiscible solvents is very rigid in organic solvents (enzyme memory) (10,20). When the lipases were lyophilized at lower or high pH (pH 3, 4, and 5 or 8, 9, and 10), the unfavorable conformation of lipase at these pH did not go back to a favorable conformation in the organic solvent, since the ionization states did not change between the ionogenic groups in isooctane. This is the reason that the lipases dehydrated at the lower and high pH had no activities was because the enzymes still maintain their denatured conformation. In fact, the pH memory exhibited in isooctane disappears in water; in contrast to organic solvents, the enzyme's ionogenic groups can readily change their ionization states (10).

Most of the enzymes were not as active over 40°C; however, the synthetic activity for Lipase PS increased up to 60°C, while *R. miehei* lipase was not as active over 40°C (Fig. 3). It was not consistent with the data of Claon and Akoh (15) where most of lipases had high activity around 80°C in terpene esterification. Klibanov (6) already reported that the lipase was not deactivated when it was stored at 120°C with-

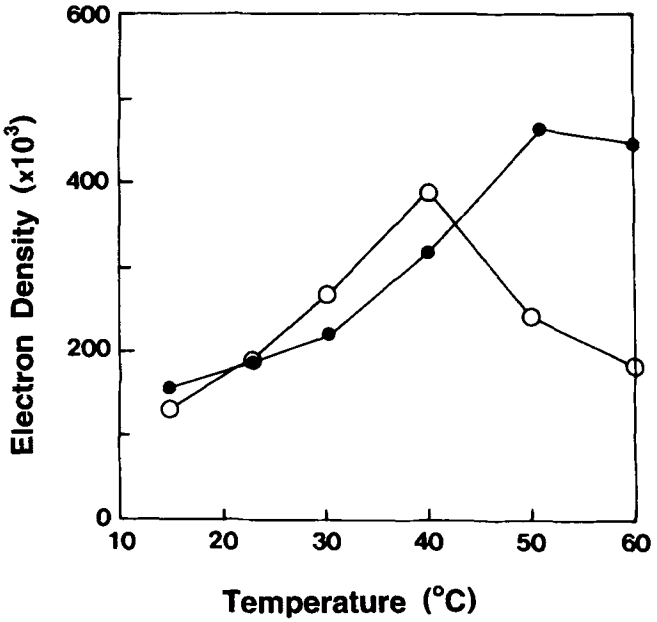

FIG. 3. Effect of reaction temperature on the synthesis of total medium-chain glycerides (tricaprin, dicaprin, and monocaprin) in isooctane by lipases from *Rhizomucor miehei* (○) and *Pseudomonas aeruginosa* (●).

out water. During the esterification of fatty acid and glycerol, water is produced from condensation of the two groups; this might act as a bridge for the exchange of ionization groups. Thus, the conformation of lipase may not be so rigid that the structure of lipase can be destroyed at high temperature. Again, it can be concluded that the thermal stability of lipase for aliphatic acid and alcohol esterification is dependent on the nature of the alcohols and acids used and varies from one enzyme to another.

REFERENCES

1. Babayan, V.K., Medium-Chain Triglycerides and Saturated Lipids, *Lipids 22*:417–426 (1987).
2. Timmerman, F., Medium-Chain Triglycerides, The Unconventional Oil, *International Food Ingred. 3*:11–18 (1993).
3. Chiang, S.H., K.H., Huang, and H.F. Lee, Effects of Medium-Chain Triglycerides on Energy Metabolism, Growth and Body Fat in Broilers, *J. Chinese Animal Sci. 19*:11–19 (1990).
4. Captex, *Product Brochure,* Capital City Products, Columbus, 1986.
5. Kim, S.M., and J.S. Rhee, Production of Medium-Chain Glycerides by Immobilized Lipase in a Solvent Free System, *J. Am. Oil Chem. Soc. 68*:499–503 (1991).
6. Klibanov, A.M., Enzyme Work in Organic Solvents, *Chemtech 16*:354–359 (1986).
7. Dudal, Y., and R. Lortie, Influence of Water Activity on the Synthesis of Triolein Catalyzed by Immobilized *Mucor miehei* Lipase, *Biotechnol. Bioeng. 45*:129–134 (1995).
8. Kwon, S.J., S.T. Kang, J.J. Han, Y.P. Lee, S.Y. Lee, M.K. Kim, D.H. Ham, and J.S. Rhee, Reactor Design for Enzymatic Production of Surfactants and Monoglycerides, KAIST Report, Seoul, Korea, 1992.
9. Zaks, A., and A.M. Klibanov, Enzymatic Catalysis in Nonaqueous Solvents, *J. Biol. Chem. 263*:3194–3201 (1988).
10. Zaks, A., and A.M. Klibanov, The Effect of Water on Enzyme Action in Organic Media, *Ibid. 263*:8017–8021 (1988).
11. Kim, K.H., D.Y. Kwon, and J.S. Rhee, Effects of Organic Solvents on Lipase for Fat Splitting, *Lipids 19*:975–977 (1984).
12. Cooper, T.G., *The Tools of Biochemistry,* John Wiley & Sons, New York, 1977, p. 189.
13. Kwon, D.Y., Synthesis of Stereoisomer Trifluoroethylmandelates and Their Stereospecificity for the Uses as the Substrate of Lipases in Organic Solvent, *J. Korean Chem. Soc. 36*:125–130 (1992).
14. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall, Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem. 193*:265–275 (1951).
15. Claon, P.A., and C.C. Akoh, Lipase-Catalyzed Synthesis of Terpene Esters by Transesterification in *n*-Hexane, *Biotechnol. Lett. 16*:235–240 (1994).
16. Yee, L.N., C.C. Akoh, and R.S. Philips, Terpene Esterification by Lipase-Catalyzed Transesterification, *Ibid. 17*:67–70 (1995).
17. Macrae, A.R., Microbial Lipases as Catalysts for the Interesterification of Oils and Fats, in *Biotechnology for the Oils and Fats Industry,* edited by C. Ratledge, P. Dawson, and J. Rattray, AOCS Press, Champaign, 1984, pp. 189–198.
18. Foglia, T.A., K. Petruso, and S.H. Feairheller, Enzymatic Interesterification of Tallow-Sunflower Oil Mixtures, *J. Am. Oil Chem. Soc. 70*:281–285 (1993).
19. Kwon, D.Y., K.H. Kim, and J.S. Rhee, Characteristic of Lipases in Two-Phase System, *Korean J. Appl. Microbiol. Bioeng. 15*:43–48 (1987).
20. Zaks, A., and A.M. Klibanov, Enzyme-Catalyzed Process in Organic Solvents, *Proc. Natl. Acad. Sci. USA 82*:3192–3196 (1985).

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